Somatic embryogenesis in sweet potato (Ipomoea batatas, L.) in relation to cryopreservation and synthetic seed production

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SOMATIC EMBRYOGENESIS IN SWEET POTATO (Ipomoea batatas, L.) IN RELATION TO CRYOPRESERVATION AND SYNTHETIC SEED PRODUCTION

submitted by

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Dedication

To the memories of my father Sultan Mahmood Bhatti and my mother Fatima.

To my wife Rukhsana H. Bhatti, my son Arsal Hussain Bhatti, Hannah with love Krisna, my brother M. Aziz Ullah, sister Nasim Fatimah, my father in law Malik Riaz-ur-Raliman.

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Abstract

Somatic embryogenesis can play an important role in sweet potato improvement programmes by providing a target tissue for the development of genetic transformation and cryopreservation protocols, and for synthetic seed production. The studies showed that embryogenic tissue could be reliably initiated from buds of the eight genotypes that were tested with frequencies ranging from 24 to 67%, using MS medium supplemented with 5μM 2,4-D or 2,4,5,-T. The embryogenic tissues could be maintained by monthly subculture to a medium of similar composition, and plant regeneration was achieved by transfer to a medium containing 1μM 2,4-D or 1μM GA_3.

In the cryopreservation studies embryogenic aggregates 1.0-2.0mm in diameter, were encapsulated in alginate gel, precultured on medium containing elevated levels of sucrose and dehydrated by evaporation prior to rapid or two-step freezing processes. The maximum survival rates were obtained with the two-step freezing process (ambient temperature to 0°C at 10°C min^{-1}, 0°C to –40°C at 0.5°C min^{-1}). This was followed by transfer to liquid nitrogen for 1h. Survival was achieved with all of the eight genotypes, with five genotypes showing rates in excess of 67%, all dehydrated to a moisture content with range 11-34%. However, the most effective sucrose pre-treatment varied with genotype.

Survival was also achieved with non-encapsulated embryogenic tissues, these were pre-cultured for a total of 8days on a series of MS media supplemented with stepwise increase in sucrose concentration up to 0.7M prior to rapid-freezing and a gradual decrease in the sucrose concentration after thawing. Among the four genotypes tested,

survival rates ranged between 33% and 81%, all after a 1.5h evaporative dehydration period. This technique has considerable advantages in terms of technical simplicity. Encapsulated shoot buds (0.2-1.0mm in length) were recalcitrant when subjected to the cryopreservation protocols that were successfully employed with the embryogenic tissue. Success was achieved, however, when a 0.4M sucrose / 2.0M glycerol mixture was added to the sucrose pre-treatment sequence. Survival rates ranging from 10 to 33% were obtained with the four sweet potato genotypes tested, all after 5h dehydration following the two-step freezing process. None of the shoot buds from any of the genotypes survived the rapid-freezing process.

Abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
2,4,5-T	2,4,5-trichlorophenoxyacetic acid
BAP	6-benzylaminopurine
CIP	International Potato Centre, Lima-Peru
d	days
DMSO	Dimethylsulphoxide
EG	Ethylene glycol
FAO	Food and Agricultural Organization
GA_3	Gibberellic acid
h	hours
IBA	Indolebutyric acid
IPGRI	International Plant Genetic Resources Institute, Rome-Italy
IAA	Indole acetic acid
IITA	International Institute of Tropical Agriculture, Ibadan-Nigeria
MC	Moisture content
MS	Murashige and Skoog Medium (1962)
NAA	α-napthaleneacetic acid
PAR	Photosynthetically Active Radiation
rpm	revolutions per minute
RF	Rapid-freezing
SF	Slow-freezing or two-step freezing

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1. GENERAL INTRODUCTION

1.0 GENERAL INTRODUCTION

1.1 SWEET POTATO

1.1.1 ORIGIN AND DISTRIBUTION

Sweet potato (*Ipomoea batatas L. (Lam)*) is a dicotyledenous perennial root-tuber plant of the family Convolvulaceae. There are 50 genera and 1200 species of Convolulaceae (Henderson et al 1984).

The origin of the sweet potato is obscure and it is unknown in the wild state. There seems little doubt that it originated in tropical America, but exactly where and by what parents is unknown with any certainty. The tuberous roots of the plant are said to have been first mentioned by an author named Pigafetta (Nicholls 1906) who visited Brazil in the year 1519 and found them in use among the Indians as an article of food. It is therefore speculated to be a native of South America. Long before Europeans arrived in this hemisphere, the Incas of South America and the Mayans of Central America grew several cultivars. One cultivar was grown for food and the other cultivars were used to supply their artists with colours for use in paints. Early Spanish explorers are believed to have taken the sweet potato to the Philippines and East Indies; from there, it was carried to India, China and Malaysia by Portuguese explorers. The sweet potato was apparently introduced into Japan from China at around 1700 by way of Ryuku Island (Boswell and Bostelman 1949).

Sweet potato is adaptable to a wide range of climatic and soil regimes. It is normally grown from 40° N to 32° S and from sea level to 3000m elevation. It grows best where the average temperature does not fall below 24°C with a well-distributed annual rainfall of 7500-1250mm and an abundance of sunshine. The plant is very sensitive to frost,

requiring a minimum frost-free growing period of 4-6 months. Temperatures below 10°C can damage the crop.

Sweet potato is quite resistant to drought, but the yield can be seriously reduced, if severe water stress occurs during the period of storage root development (Kassan 1976).

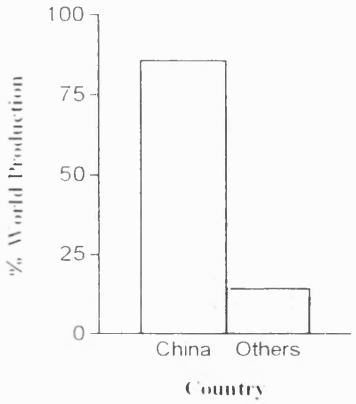
1.1.2 Economic Aspects

1.1.2.1 Production levels

The sweet potato is an important root and tuber crop in developing countries and ranks seventh in terms of worldwide staple food production (Horton 1989). According to the FAO (1993), it is grown in tropical, subtropical and warm temperate regions of the world covering approximately 9 million ha., and has with a total world annual production of approximately 12 billion tonnes. The average yield of sweet potato across the world is 13.6 tonnes/ha. China is the chief sweet potato producer, accounting for about 85% of world production followed by Indonesia, Vietnam, Uganda, Japan, India, Brazil, Philippines, Tanzania, and Pakistan (Fig. 1).

In Pakistan, the roots of sweet potato are used for human consumption and the foliage is used as forage. The area in Pakistan planted with sweet potato is 1000 ha which represents 0.01% of the world crop. However, the yield per hectare in Pakistan is 124% of the average world yield because of suitable climatic and soil conditions. Therefore, the potential for increased production of sweet potato is considerable. Pakistan already generates income from the export of fruits and vegetables to the Middle East and European Countries and sweet potato could contribute towards this particular market.

a) China and other producers



b) Other major producers

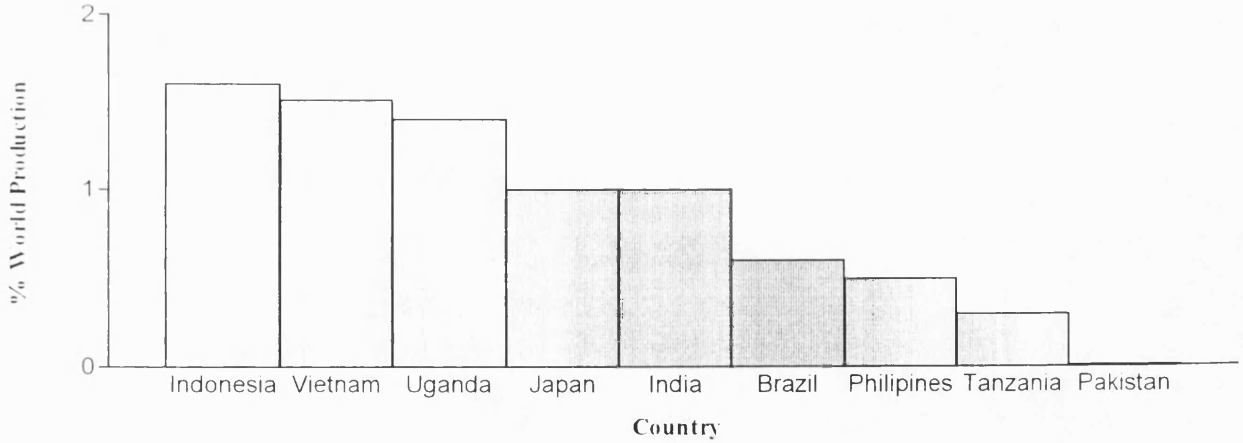


Fig.1. The world major producers of sweet potato.

1.1.2.2 Nutritional Value

Sweet potato is a valuable source of nutrition and sustenance to people especially in the developing world (Walter et al 1984). It is an important source of carbohydrate, with a high calorific value, and it can provide about 113 calories per 100g, whereas potato provides only 75 calories, although despite the caloric difference potato may elevate blood sugar levels more than sweet potato (Martin, 1984). The tuber contains approximately 20% starch and 5% simple sugar and is generally considered to be a high-energy food, and in some parts of the world it is the staple crop. It also provides a considerable amount of vitamin C (20-30mg/100 gm.) and deep yellow varieties of sweet potato can provide sufficient pro-vitamin A carotenoids for health, if eaten in quantity, although, the overall variation between varieties in carotene content is great (0-8000 Iu/100g). Vitamin B_1 (thiamine) is also present in adequate amounts in relation to the calorie content (0.8-1.0mg /100kcal.); this amount is about twice the level required by humans. Potassium (200-300mg /100g) is known to be the predominant mineral present and the iron content (0.8mg /100g) is sufficient for those sweet potato eaters who consume 2kg or more per day. However, sweet potato is a starchy root and it contains very little fat (0.1-0.2%). The content of protein (mainly in the form of a kind of globulin) is also generally low, providing about 4-6% of the total calories (1-2% on a wet weight basis), although the essential amino acid pattern is quite good and the limiting amino acid is leucine (Huang, 1982).

1.1.2.3 Other uses

The raw storage roots can be eaten boiled, fried, roasted and baked or they can be processed as wine, chips, flour or starch (Onwueme, 1978). In Japan, over half of the annual production is used for wine, starch and alcohol production (Simpson and

Orgonzaly 1986). Fresh leaves and shoot tips are eaten fried, boiled or in soups and stews, and shoot tips and leaves are available throughout the year in the markets of the Philippines, Malaysia and Indonesia. (Villareal et al 1979, Martin 1984,). Medical researchers have pointed out that sweet potato leaves possess unique properties as diuretics and saliuretics (Villareal et al 1979).

Farm animals can eat the whole plants, fresh or in the form of silage. The vines can be an important source of nutrients for animals, especially in the dry seasons when sweet potato is more resistant than other forages. Special cultivars of sweet potato which produce large amounts of leaves are used as forage in some countries (Onwueme 1978, Fouda 1988).

Sweet potato is also used as a houseplant in some sub-tropical and tropical countries (Huaman and De la Puente, 1988).

1.1.3 Conventional Production and Breeding practices

1.1.3.1 Propagation and Field Practices

Sweet potato is conventionally propagated by vine cuttings as well as by tubers. If the crop is to be grown from tuber, the sets should be derived from robust, healthy tubers rather than using runts and non-marketable root pieces. The sets should be small (20-50g) and should be planted only about 3cm deep (Ikemoto, 1971). The use of sets derived from the tuber for direct planting of sweet potato is however not recommended as general practice, because it usually results in very low yields.

The use of vine cuttings is the recommended commercial method of propagating sweet potato and it is better than using tuber sets for several reasons. Firstly, plants derived from vine cuttings are relatively free from soil-born diseases. Secondly, by propagating

with the vines, the entire tuber harvest can be saved for consumption or utilization instead of reserving some of it for planting purposes. Thirdly, vine cuttings yield more heavily than sets and produce tubers of more uniform size and shape. In the use of vine cuttings, pieces from the stem apex are preferred to those from the middle and basal positions of stem (Shanmugavelu, *et al*, 1972).

The vines are normally planted 25-30cm apart on ridges that are 60-75cm apart. It is best to plant sweet potato early in the rainy season so that it has the entire rainy season in which to grow. Where the rainy season is long, planting may be delayed and timed so that the crop matures before the rainfall begins to decline.

Sweet potato is grown in various kinds of rotations around the world. In Sierra Leone, for example, it is often alternated with swamp rice or hungry rice (Digitaria). There, as in Zanzibar, the rice crop has been found to do well after sweet potato. One useful property of sweet potato in rotations is its ability to smother and control weeds. In many parts of the humid tropics, it is possible to grow two crops of sweet potato a year. In the drier areas, as well as in temperate zones, only one crop per year is possible.

Sweet potato is harvested in 3-8 months after planting, although in most of the tropics, the crop requires 5-6 months to mature. The exact duration of the crop varies with cultivar and with the environmental conditions under which it is grown. The majority of the world's sweet potato crops are harvested and stored by the use of manual practices. (Wilson and Abrams, 1982; Onwume, 1978).

After harvest, the roots are cured at 27-29°C with a high relative humidity (85-90%). In the tropics, this process occurs naturally. Curing is necessary to minimise infection by

micro-organisms and to increase resistance to damage during handling (Onwume, 1979). Storage is recommended at 13-16°C with 85-90% relative humidity. Most of the sweet potato produced in the tropics is not stored under controlled conditions and, consequently, post harvest losses of 25% are normal (Onwume, 1978, Euroconsult, 1989).

1.1.3.2 Production Constraints

The major constraints on sweet potato productivity worldwide are diseases and insect pests. Diseases are responsible for the destruction of thousands of acres each year and they cause severe losses, not only in the field, but also during storage. Field diseases account for approximately 70-80% of disease damage each year. Mosaic is a serious virus disease of sweet potato worldwide and it is caused by a strain of tobacco mosaic virus. Combinations of two out of three viruses cause the feathery mottle complex; including the internal cork virus, the leaf spot virus and white fly transmitted yellow dwarf virus. *Fusarium* wilt or stem rot is caused by *Fusarium oxysporum batatas* and other fungal diseases are also numerous, e.g. black rot (*Endoconidiophora fimbriata*), mumification (*Monilichaetes infuscans*), white rust (*Albugo Ipomoea panduranae*), stem rot (*Rhizoctonia solanai*), foliar mottle (*Phyllostica batatas*). Bacterial diseases include root-rot caused by *Streptomyces ipomoea* and warehouse rot caused by several organisms.

The sweet potato weevil, *Cylas formicarius elegantus*, is the most damaging insect pest of sweet potato in the world, followed by the larvae of the beetles *Diabrotica baltiata* and *Systena blenda*. All three insects do damage mainly to storage roots, usually causing damage to the skin surface (Henderson et al. 1984, Scaramuzzi, 1986

1.1.3.3 Breeding

Millar (1937) induced sweet potato flowering and set seed, enabling significant improvements to be made in the USA breeding programs. Because of his vast contribution to sweet potato releases, Millar is often referred to as the father of sweet potato developments (Bowers et al 1969). The aims of crop improvement in sweet potato include higher yields, resistance to diseases and pests, shorter growing season and improved tuber quality. Also, a certain amount of effort has been spent trying to produce cultivars that are less sweet, since consumers in many parts of the tropics find the extreme sweetness of certain cultivars objectionable. This is true in West Africa where for example, where fewer cultivars of sweet potato are used in comparison with yam, the more popular staple. A major problem in the breeding of sweet potato is degeneration of the sexual reproduction system as a result of photoperiodically controlled flowering, low pollen viability, short flower life, slow rate of pollen tube growth, incompatibility between complexes, poor seed set and dormancy problems. Fortunately, in view of the difficulties associated with sexual propagation, vegetative propagation of sweet potato is very straightforward and new advances in tissue culture and somatic hybridization offer great potential in relation to breeding.

The methods used in sweet potato improvements are the following:

a) Selection of clonal material: In the natural course of events, mutations arise fairly frequently in sweet potato experiments. Some of these mutations can be observed as chimeras of the tuber, bud or vine. Close scrutiny is required to identify and propagate such mutant plants or plant parts, and although most of the mutations are in the inferior direction, some of them give rise to superior characteristics. Once a superior mutant has been identified, it can be multiplied rapidly by vegetative means. New varieties have been developed by this procedure.

b) Hybridisation: Hybrids are produced as a result of controlled crossing and selection is then carried out on them. Sweet potato improvement through hybridisation must of course rely on the sexual processes, with the attendant difficulties), but any desirable individual that results can be multiplied and perpetuated with ease through vegetative propagation.

c) Mutation breeding. This implies treating the sweet potato material with artificial mutagens (e.g. X-rays, colchicine), growing the resultant mutants, and selecting for the desirable ones among them (Onwume, 1978). Again vegetative propagation techniques are a valuable adjunct because they allow the rapid multiplication of mutants for field trials and selection.

1.1.3.4 Germplasm Storage

Since sweet potato can be propagated from *rooted* vine cuttings, storage roots and rooted leaves (Martin, 1984) accessions of sweet potato are frequently maintained in field genebanks. Such field collections are however subject to rapid genetic erosion as individual accessions are exposed to various environmental stresses and to attack by pests and diseases (Fernandez et al, 1987). As a consequence, sweet potato germplasm collections in tropical areas are typically replanted and moved at 4-6 month intervals to reduce the incidence of soil-borne diseases and insects, but this procedure is labour-intensive and it exposes the collections to additional biotic and abiotic hazards. Many difficulties arise during the maintenance of germplasm in the field and most significantly germplasm can not remain virus-free for subsequent distribution (IBPGR, 1988). The advantage however of field gene bank maintenance is the simplicity of the technique and consequently it is a common method of conservation, but ideally it should be

complemented by an *in vitro* storage facility which can guarantee long term security and freedom from pests and pathogens.

1.1.4 BIOTECHNOLOGY AND SWEET POTATO

1.1.4.1 Propagation

Shoot meristem-tip and single-node cultures provide the basis for the most common in-vitro methods employed for micropropagation because these methods are widely applicable with different species, such methods have been developed for in-vitro propagation of sweet potato. In vitro plantlet regeneration of sweet potato has been reported utilizing shoot meristem-tip culture (Alconero et al, 1975), and in vitro propagation of two selections of white-fleshed sweet potato was obtained on modified MS medium using lateral buds and shoot apices as primary explants (Litz and Conover, 1978). In-vitro techniques based on shoot meristem-tip culture have the following advantages over traditional methods:

1. A large number of plants may be maintained and multiplied in a small amount of space.

2. Propagation is carried out in aseptic conditions free from pests and pathogens, and so, in combination with appropriate disease-indexing procedures, plants that can be produced in large number can be certified to be free of known pathogens (i.e. "pathogen-tested").

3. Plants can be produced all year round.

4. Pathogen-tested vegetative material can be readily stored over long periods under protected conditions ("nuclear stocks").

5. . Plants need minimal attention between subculture and therefore there is no labour or material requirement for watering, weeding, spraying etc.

The chief disadvantages of *in vitro* methods are that more advanced skills are required for successful operation, together with more specialised and relatively expensive production facilities. As a result of these requirements, production costs can be quite high compared with conventional propagation methods and therefore, the cost of propagation is likely to be uneconomically high. There is, however, scope for the development of simpler, low-cost *in vitro* facilities, as demonstrated in Vietnam for potato (VanVyen and VanderZaag, 1983), which may be able to deliver pathogen-tested propagates at economic prices to farmers. Even so, it is unlikely that such techniques would be employed on a regular basis to deliver planting material directly to the field, because of the numbers of plants required and the cost. However, in some circumstances, it can be demonstrated that it would be an advantage to receive healthy plant material on a regular basis as long as the cost is not too high.

It is possible that alternative *in vitro* could be developed for mass propagation based on the production of gel-encapsulated somatic embryos ("artificial seeds") (Redenbaugh et al, 1987), but these still require further research before these can be an economic proposition.

1.1.4.2 Plant health

The level of success in controlling diseases depends entirely upon the nature of the pathogenic agents and the exact mechanisms by which they affect the host system. Virus diseases for example, are present in virtually all root and tuber crop species and, depending upon the severity of infection and the nature of the host plant involved, this

can cause serious yield losses (Kartha, 1982). Plant tissue culture research over the last two decades has contributed substantially towards alleviating the problems of viral infection and the production of disease-free plants. The most important technique is shoot meristem culture, which has found its place not only in clonal propagation but also in the production of plants free of diseases; especially those caused by viruses. The inherent genetic stability of shoot apical meristems and features of their morphology make them ideal material for the long term storage of pathogen-tested germplasm and its international exchange (Kartha, 1984).

An alternative approach to improve plant health quality in sweet potato would involve the development of new cultivars with high levels of resistance to important pests and pathogens. However, very limited investigations have been made with other related species or genera as a basis for transferring desirable resistance genes into *Ipomoea batatas*. Incompatibility has caused numerous problems in hybridizing potentially desirable parents from within the species and this is an even greater problem with regard to inter-specific on inter-generic hybridization. New advances in *in-vitro* techniques, including somatic hybridization, however, offer possible ways of overcoming these problems but, to date, this area has received limited investigation.

It is possible that transgenic techniques might be used for production of improved cultivars and *in-vitro* techniques, especially those involving somatic embryogenesis, are likely to be used as a key component of the genetic transformation protocols.

1.1.4.3 Genetic conservation

Genetic conservation is the process of preserving the genetic variability of crops for the future use in plant breeding programs. The International Board has taken an important

initiative in the area of genetic conservation for Plant Genetic Resources (IBPGR). Recognising the potential benefits of the approach to the conservation of problem material (Withers and William, 1982), the IBPGR established an Advisory Committee on Storage to monitor developments in the relevant technologies and recommend areas where research emphasis would be potentially beneficial (IBPGR, 1986). Methods for the maintenance of *in vitro* collections depend upon the biology of the crop concerned. In the last twenty years, attempts have been made to develop methods that can reduce or stop the growth of shoot cultures in order to reduce the maintenance costs and maintain genetic integrity. These methods include the application of reduced-growth procedures based on the use of sub-optimal temperatures and/or high osmolarity media (Henshaw, 1987).

To date a limited number of reports have been published describing methods that have been tested in research related to the conservation of sweet potato germplasm. Allan (1979) conserved nodal cuttings at reduced temperatures varying from 6 to 28°C. The best results were obtained at 22°C where plantlets were still alive after 55 weeks. Jarret and Gowell (1991) added 0.01 to 10 mgl^{-1} ABA to basic medium (MS) and maintained the cultures at 28°C. The results showed complete inhibition of growth with 95% viability after 90,180 days and 1 year of culture on media containing between 1 to 10mgl^{-1} ABA.

Cryopreservation is an ideal alternative system for genetic conservation since, in theory, genetic stability is guaranteed over very extended storage periods which involve relatively low maintenance (Withers, 1988). Cryopreservation is based on the arrest of metabolic functions of biological material by the reduction in temperature to that of liquid nitrogen whilst maintaining viability. At the temperature of liquid nitrogen (-

-196°C), the metabolic activities of cells are at a standstill and they can be preserved in such a state for many years. Also there is, in theory, little risk of genetic instability, except as a result of background radiation. Cryopreservation techniques are described in more detail in section 1.3.

1.2 SOMATIC EMBRYOGENESIS

1.2.1 HISTORY, THEORY AND DESCRIPTION

Somatic embryogenesis is the production asexual or adventitious embryos from somatic cells, usually under *in vitro* conditions. The phenomenon was first observed in suspension cultures of carrot (*Daucus carota*) by Steward *et al*, (1958) and in carrot callus by Reinert (1959), and subsequently carrot has been widely used as a model system for the study of somatic embryogenetics. The somatic embryo is characteristically formed as an independent bipolar structure and it is not strongly attached to the tissue of origin (Haccius, 1978). Such an embryo can further develop and germinate into a plantlet through a sequence of events similar to that followed by the zygotic embryo inovule. Production of somatic embryos from *in vitro* systems may occur either directly or indirectly. The direct mode of somatic embryogenesis involves the formation of embryos, from a single cell or a group of cells, on a part of the explant tissue without an intervening callus phase (Raghavan, 1976 and Tesserat et al, 1979 Karpoff. 1982). More commonly somatic embryos are formed indirectly from cells that have proliferated following the isolation of the original explant. Although the development of somatic embryos closely resembles that of zygotic embryo, one feature of somatic embryo development is the continuation of cell division resulting in embryos larger than their zygotic counterparts. When the cotyledons start to grow, they form a broad disk-shaped meristem with many cell divisions visible around the periphery and in the centre. The

cotyledons often appear fused along their margins producing a fasciated tube-like structure rather than separate distinct cotyledons. Also, there may be premature cell enlargement and differentiation of somatic embryos. In the normal zygotic embryo of carrot, the cells remain small, dense, cytoplasmic and undifferentiated apart from changes in shape. In somatic embryos on the other hand, highly vacuolated cells may be clearly visible as shown by absence of intense Feulgen-staining, especially in the hypocotyl region. Also, in somatic embryo development, altered spindle orientation, early cell differentiation and delayed initiation of cotyledons and shoot apex can occur (Ammirato, 1987). Haccius (1978) defined a non-zygotic embryo as a new individual arising from a single cell and having no vascular connection with material tissues. Raghavan (1976) and Tisserat *et al* (1979) were more cautious in recognising that a single cell origin had not been unequivocally demonstrated in many cases where apparently normal bipolar somatic embryos were formed from aggregates of cells. In addition to the primary somatic embryos that are derived from the initial explant or from proliferated tissues, there are also secondary somatic embryos that are produced from the tissues of the primary embryos (Litz and Conover, 1983).

1.2.2. Induction of Somatic Embryogenesis

1.2.2.1 General

Under *in-vitro* conditions, somatic embryos are produced either directly from the cells of the original explant or directly from cells which have proliferated from the explant cells (Williams and Maheswaran, 1986). Sharp et al (1982), distinguished between two types of embryogenic response : (I) direct embryogenesis in which "pre-embryogenic determined cells" (PEDC's) present in the explant responding to permissive conditions divide to produce somatic embryos without an intervening callus phase, and (ii) indirect embryogenesis, in which differentiated cells are stimulated to divide and de-differentiated

to form "induced embryogenic determined cells" (IEDC's), from which the somatic embryos again develop under permissive conditions. It is likely that the two pathways operate according to the conditions and the nature of explant. Regardless of the pathway, there is general agreement that the embryogenic tissues consist of small tightly packed cells with dense cytoplasmic contents and that these cells are quite unlike the large vacuolated cells commonly found in callus and suspension cultures.

In carrot (Becks-Huseman and Reinert, 1970) it has been found that somatic embryos can be derived each from a single original somatic cell, and this can result in the formation of a large number of embryos in cell suspensions or rather fewer embryos in callus. The embryogenically determined cells, once they have been induced to divide, generally from distinctive globular structures, which can proliferate in this form or give rise to somatic embryos according to conditions. Although, it has been suggested that these embryos can arise from a single cells (Becks-Huseman, Reinert, loc.cit., Button et al, 1974), it is usually not possible to say whether there has been some proliferation of the embryogenic cells before the embryo is actually formed.

The development of somatic embryos closely resembles that of zygotic embryos morphologically and this is possibly suppressing, considering that somatic embryos develop completely outside both the physical constraints and informational context of maternal tissue. The fact that a structurally and developmentally normal embryo can develop from a somatic cell clearly indicates that the main features of embryo development are under the control of mechanism located within the embryo itself.

Overall, it is apparent that the process of somatic embryogenesis is affected by many factors that can operate either individually or in combination according to circumstances:

(I) Formulation of medium

The general formulation of the basal medium has been shown to have an influence on somatic embryogenesis. With regard to formulation of media, Ling et al (1983) suggested that Murashøge and Skoog medium is the most suitable medium for the high frequency induction and maintenance of the embryogenic callus in rice, while Heller's medium is more suitable for development of somatic embryos. It was also noted that the effect of White's medium on the embryogenic process was similar to that of MS medium, if extra KNO_3 was added to the former.

Other studies have shown that sources of nitrogen in the medium affect somatic embryogenesis of carrot in ways which are dependant upon the species and auxin concentration (Halperin, 1965; Reinert, 1967; Raghavan, 1976). The effects of amino acids such as L-glutamine and tryptophan and mixtures such as casein hydrolysate and yeast extract on embryogenesis have been demonstrated. Litz et al (1982) suggested that glutamine addition to the medium helped to induce somatic embryogenesis in *Mangifera indica*; in contrast, the addition of glutamine or proline to the callus induction medium did not increase the frequency of embryogenic callus formation from stem explants. In all studies, there was nitrate in the medium. In these cases, however, ammonium ion served as the sole nitrogen source (Dougall and Verma, 1978).

Another important component of medium shown to influence embryo induction is the concentration and type of sugar present (Strickland et al, 1987; Lazzeri et al, 1988). Lu et al, (1982) found that a concentration of $0.5mg^{-1}$ 2,4-D with a high concentration of sucrose (12%w/v) was the most suitable for induction of embryogenic callus in corn. Eapen and George (1993) stated that among the different sugars tested, sucrose (6% w/v) was the most favourable for somatic embryo induction in peanut, followed by fructose and glucose, while maltose was completely ineffective. The most commonly used

carbohydrate for plant tissue culture is sucrose. In contrast, however with *Medicago sativa*, maltose induced the highest embryo yield (Strickland et al, 1987).

(ii) Concentration and type of auxin

The pioneering work of Steward and collegues (Steward, 1968) showed that out of a number of auxins tested, 2,4-D was the most effective for inducing somatic embyogenesis from the more mature carrot tissues, whereas the choice of auxins was less critical with the tissue from seedlings and embryos. Subsequently, in a review of somatic embryogenesis studies over a period of some twenty years following Steward's early work, Sharp et al (1982) concluded that 2,4-D had been the auxin of choice in the largest proportion of reports.

These general conclusions about the importance of 2,4-D have been further confirmed in a number of detailed studies of the effects of different auxins on somatic embryogenesis. For example, Eapen and George (1993) stated that among the different auxins tested, 2,4-D was the most effective in the production of somatic embryos in peanut.

Despite these results, however, a number of important crop species or particular genotypes within a species have been found to be recalcitrant with the auxins most commonly used plant tissue culture studies. Auxins such as IAA, NAA and 2,4-D have been employed for the induction of somatic embryogenesis and with some of these species, greater success has been achieved with other less common used auxins such as Picloram, dicamba and 2,4,5-T (Eapen and Rao, 1982; Ozias-Akins et al, 1992; Eapen and George, 1993).

(iii) Genotype

It has been a frequent observation that the induction of somatic embryos varies not only from species to species but also from genotype to genotype. For example, Van-Doorne et al

(1995) screened 16 cultivars of pea from induction of somatic embryos. Three cultivars showed the highest tendency to form somatic embryos while a further three cultivators did not show any embryo production at all and the other cultivars showed intermediate responses.

iv) Explant source

The early work of Steward and colleagues with carrot clearly showed that embryonic competence varies considerably with maturity of tissues and according to the regions of the plant form which they have been isolated (Steward, 1968). These results have been confirmed in numerous other studies and, in some of the more recalcitrant species, the competent tissues can be extremely localized in distribution e.g. cassava (Stamp and Henshaw, 1987). In general, however, the more immature tissues in embryos and seedlings and the merismaic regions of more mature plants are found to be the more likely sources of embryogenically responsive explants. In some species such as carrot, the relatively wide occurrence of embryogenically competent tissues in different regions of the plant (Steward, loc; cit.) might suggest that somatic embryos can be derived from both PEDC's and IEDC's (see section 1.2.2.1). However in other species in which embryogenically competent tissues have only been identified in mature plants may be a more permanent state

1.2.2.2 Induction of somatic embryogenesis in sweet potato

Somatic embryogenesis offers significant potential for the improvement of sweet potato as a system to generate tissues for genetic transformation and for mass clonal propagation based on synthetic seed production. For these purposes, however, it is essential that the techniques can be applied to a wide range of cultivars. Cantliffe and his co-workers have carried out extensive investigations of somatic embryogenesis in sweet potato with a

single cultivar (cv. White Star). In these studies, it was shown two types of callus tissues were produced from shoot-tips in the presence of 5μM and 10μM 2,4-D. The first type of which was non-embryogenic and which occurred within two weeks was friable, somewhat uniform in structure, translucent, white to brownish in colour and it was made of large vacuolated cells (50-200μm in diameter). The second type, which occurred after 2-6 weeks, was firm with an irregular and nodular surface and it was opaque and yellow in appearance. This tissue was embryogenic and it was formed from smaller interconnected callus units, made of densely cytoplasmic cells (10-20μm in diameter). The origin of the yellow embryogenic callus within the explant was not determined. After subculture, the peripheral layers of the yellow callus continued to proliferate while the inner tissues and the tissue in contact with medium became mucilaginous (Chee and Cantliffe, 1988; Cantliffe et al, 1988 (a)). In all of the work, however, the evidence for the induction of somatic embryogenesis at higher frequencies has been restricted to one cultivar. When attempts have been made to be extend this to a wider range of genotypes, many have been found to be recalcitrant or they have responded at low frequencies, generally less than 20% (Prakash and Varadarajan, 1992).

Despite the extent of these studies, therefore, it is apparent that it is not possible to transfer the conclusion to other sweet potato cultivars (Al-Mazrooei, 1996). The studies showed that out eight genotypes investigated, three produced somatic embryos, with induction rates between 32% and 80% and three genotypes completely failed to produce somatic embryos.

In order for this important tropical root crop to benefit from improvement through the application of bio-technology, it is essential that efficient culture systems are developed

which allow somatic embryogenesis to be effectively induced and maintained in as wide a range of genotypes as possible.

1.2.3 APPLICATION OF SOMATIC EMBYOGENESIS

1.2.3.1 Micropropagation

Micropropagation is normally based on the stimulation of multiple-shoot growth from cultured shoot-tip and nodal explants (Murashige, 1974; Brown and Thorpe, 1995). It has not always been possible to adapt this approach for the routine propagation of certain crop species, because the proliferation rate thus obtained may be too low for it to have practical utility and cost effectiveness; examples would include many of the commercial tree species and most of the important field crops (Rao, 1977). Such propagation methods, however, provide very much more rapid multiplication rates than conventional propagation methods and increasingly, they are being used for commercial purposes. In addition to this use for rapid propagation, the basic micropropagation methods have at least two further applications of commercial significance :I) to eliminate systematic pathogens, particularly viruses from vegetatively-propagated plants, ii) to conserve pathogen-tested stocks of vegetatively-propagated plants, particularly germplasm collections of crop plants needed for plant breeding and nuclear stocks required for phytosanitation programs. Alternative micropropagation methods based on somatic embryogenesis have therefore been under investigation for species such as oil palm (Jones and Hughes, 1989). These species do not respond to shoot-tip culture techniques, and which, it is believed, would benefit from the availability of a practical clonal propagation system. The advantage of a mass propagation technique based on somatic embryogenesis is that the embryos can be processed to produce artificial or synthetic

seeds which have many of the features of conventional seeds and production methods may be more readily automated.

Two types of artificial seeds have been proposed :I) hydrated, and ii) desiccated. Hydrated artificial seeds consist of somatic embryos individually encapsulated in a hydrogel such as calcium alginate. The embryos are first mixed with sodium alginate and then dropped into a calcium salt solution to form calcium alginate beads which are washed in water and then planted (Redenbaugh et al, 1986). Desiccated artificial seeds have been produced by coating a mixture of carrot somatic embryos, roots and callus in polyoxyethylene glycol before drying for several hours on a Teflon surface in a laminar-flow cabinet. Relatively low survival rates (3%) were, however, recorded after rehydration of the desiccated embryos, but uncoated embryos after drying showed no survival (Kitto and Janick, 1985a; 1985b). Generally speaking, hydrated capsules are difficult to store because of the respiratory requirements of metabolically active embryos and the tendency of the hydrated capsules to dry out quickly unless kept in a humid environment or coated with hydrophobic membrane (Redenbaugh, 1987b). On the other hand, desiccated artificial seeds have different problems that need resolution. The desiccation process itself can damage the embryos, although Gray (1987b) found that desiccation appeared to release grape somatic embryos from the quiescent state, thus producing improved germination. However, no coating method was used and embryo germination decreased rapidly with storage. Considerable progress has been made with alfalfa somatic embryogenesis which can now be highly controlled and uniform, with the result that high quality embryos can be produced routinely from several genotypes (Brown and Atanassov, 1987; Strickland et al, 1987).

Less attention has been given to the possibility of encapsulating non-embryogenic invitro derived vegetatively propagules. Nevertheless, some authors have tried to encapsulate shoot-tips or axillary buds of different species with promising results (Ganapathi et al, 1992; Bapat, 1993). This kind of capsule could be useful for germplasm conservation and for the exchange of sterile plant material between laboratories (Accart et al, 1994). For more general purposes the theoretical advantages of methods based on somatic embryogenesis rather than other invitro propagules are that much higher propagation rates can be achieved under conditions which can be automated. There is, however, a risk that embryogenesis propagation systems are more unstable genetically and this would require investigation for any proposed commercial system.

Somatic embryogenic tissues of sweet potato offer opportunities for micropropagation and development of synthetic seed production technology. Application of somatic embryogenesis is required and abundance of embryogenic callus for synthetic seed. A series of reports have been published which detail the induction of embryogenic tissues of sweet potato (see section 1.2.2.2). In past studies, mass production of embryogenic tissues to one cultivar or few cultivars has been difficult because of recalcitrant cultivars. More recently, an efficient invitro plant regeneration system characterised by rapid and continuous production of embryogenic tissues from leaf and petiole explants has been developed in sweet potato genotype PI318846-3 (Zheng et al, 1996). The success of synthetic seed system would be required to produce good quality and quantity of somatic embryos and to give a wide range of genotypes in sweet potato.

1.2.3.2 Genetic transformation

Techniques for the transfer of foreign genes into plants are generally dependent on the availability of an efficient invitro regeneration system. Transformation can be achieved by several methods including insertion of DNA into protoplants by microinjection

(Crossway et al, 1986), electroporation (Horn et al, 1987). The most significant progress has resulted from the development of techniques for the microprojectile bombardment of regenerative tissues by DNA-coated tungsten or gold particles (Klein et al, 1987; Sanford , 1989) and the use of genetically engineered virulent strains of Agrobacterium tumefacian as vectors (Herrera- Estrella et al, 1983; Herrera-Estrella and Simpson, 1995).

In comparison with organogenic procedures, the invitro regeneration of transgenic plants via somatic embryogenesis has some theoretical advantages such as the efficient production of large numbers of regenerants. As they are more likely to be of single-cell origin, may include a lower proportion of chimera plants (Ammirato, 1983; Sato et al, 1993). Therefore, the somatic embryo system is being increasingly employed to produce transgenic plants (Ritchie and Hodges, 1993). Dispensing the embryogenic cells in the form of suspension cultures can also increase the efficiency. This has been particularly important in species which do not respond to the Agrobacterium vector systems .g. cereals (Fromm et al , 1990; Christou et al, 1991; Vasil et al 1992) or species which do not readily undergo organogenesis e.g. Cassava (Taylor et al, 1996).

The introduction of single gene traits into sweet potato via conventional breeding is complicated due to the hexaploid nature of the plants. Genetic engineering techniques, therefore, should offer a means of directly transferring genes into sweet potato that confer resistance to various pests and diseases thereby circumventing the need for an extended crossing and backcrossing program.

When the present work started, some progress had been made in the production of transgenic sweet potato plants using different gene transfer systems including Agrobacterium rhizogenes and A. tumefaciens (Dodds et al, 199; Al-Jaboory et al, 1991). Recently, progress has been made with techniques involving electroporation

of protoplasts (Nishiguchi et al, 1992), particle bombardment (Prakash and Varadarajan, 1992) and Agrobacterium tumefaciens (Newell et al, 1995; Maria et al, 1996).

1.2.3.3 Genetic conservation

Most plant species produce long-lived ('orthodox') seeds, which under appropriate conditions, are particularly suitable for the long-term conservation of germplasm. For a number of species (e.g. sweet potato) however, it may be necessary to store germplasm in the form of vegetative propagation because of a need to conserve clonal genotypes. Other species produce short-lived ('recalcitrant') seed that are not suitable for long-term storage (Ellis et al, 1990). For most of these species, despite extensive seed research there seems to be no alternative to the use of conservation methods based on vegetative propagules. There is now general consensus that invitro methods, preferably using shoot-meristem cultures, have a key role to play in the conservation of vegetative germplasm (Henshaw, 1980).

It is recognised, however, that certain species particularly some of the woody species that produce recalcitrant seeds, do not readily initiate shoot-meristem cultures. For these species, it is necessary to consider the use of alternative invitro systems for the storage of either isolated zygotic embryos or somatic embryos (Engelmann et al, 1995).

With all of these species, the ideal invitro storage systems involves complementary restricted-growth and arrested-growth (cryostorage) techniques, with the later being particularly important for both zygotic and somatic embryos. Somatic embryogenesis in combination with cryostorage techniques, may, therefore have a specialised role to play in relation to the genetic conservation of certain plant species and this is discussed further in section 1.1.4.3.

As far as sweet potato is concerned, there is no obvious genetic role for somatic embryogenesis but cryostorage techniques were studies with embryogenic tissue in the

present investigation. This was because of difficulties encountered with shoot-meristem as well as the need to store embryogenesis lines of tissues used in genetic transformation programs (see section 1.2.3.2).

1.2.4 LONG-TERM MAINTANANCE OF EMBRYOGENIC CULTURES

Embryogenic cultures are usually maintained on a medium similar to the induction medium, which typically includes an auxin. The method of maintenance is usually based on the need to encourage a state of rapid growth, at the same as preventing the development of either mature embryos or non-embryogenic tissue. Several reports have been published which detail the maintenance of embryogenic cultures and several factors seem to play an important role, including growth regulators and subculture regimes. Medium supplemented with 2,4-D alone has been used to maintain embryogenic carrot cultures (Reinert et al, 1968; 1970), papaya cultures (Litz and Conover, 1983), and sugarcane cultures (Chen et al, 1987). Embryogenic cultures of other plant species such as gladiolus (Stefaniak, 1994) have been maintained on MS medium containing 2mg/l 2,4-D or 10mg/l NAA and 3% sucrose. Friable, non-embryogenic and embryogenic callus was selected and maintained on fresh medium and rice cultures were maintained in medium with combination of 2,4-D and cytokinin. Liquid culture has been shown to be more suitable for maintaining embryogenic conifer cultures in a rapidly growing state for large scale propagation and mechanically stirred or airlift bioreactors are the ideal vessels for producing uniform growth (Tautorus et al, 1991). However, bioreactors are complicated and expensive and costs would be considerable where a large number of genotypes are to be maintained. Bioreactor systems, therefore, may offer insufficient advantage over the simpler batch-culture systems, which are routinely subcultures before carbohydrate or other nutrients becomes limiting (Lulsdorf et al, 1992). These authors

showed that liquid cultures maintained in flasks are capable of sustained rapid proliferation but are prone to swift changes in embryogenic competence and viability, so a reserve supply of somatic embryos is required to re-establish the liquid culture should it fail.

There have been some reports on the long-term maintenance of embryogenic cultures of sweet potato. Eight separate embryogenic lines of the genotype 'White Star' were maintained for 34 months on semi-solid MS medium containing 10μM 2,4-D and 1μM BAP and in liquid medium containing 5μM 2,4-D (Chee and Cantliffe, 1988). Embryogenic callus lines from five genotypes of sweet potato were maintained on basal MS medium supplemented with 10μM 2,4-D for 12 months without any apparent loss of embryogenic competence (Al-Mazrooei, 1996).

In general, the long-term maintenance of embryogenic competence depends on the avoidance of conditions which allow the auxin concentrations to fall below a level at which embryo development is inhibited or which allow the highly vacuolated non-embryogenic cells to proliferate at the expense of the embryogenic tissue. This is most readily achieved by frequent subculture in combination with vigorous visual selection to exclude the non-embryogenic tissues.

1.2.5 Maturation and Germination of Somatic Embryos

Ultimately, the successful initiation of embryogenic cultures in plant improvement programmes depends on the efficient recovery of plants from the somatic embryos. The type and concentration of auxin used in somatic embryo induction medium influence the conversion of somatic embryos into plantlets. The best plant conversion frequency (25%) with the embryogenic cultures of peanut derived from cotyledon explants was obtained when dicamba (22.6μM) or NAA (45.2μM) were used for somatic embryo induction

rather than 2,4-D and 2,4,5-T (22.6μM) (Eapen and George, 1993). Sellars et al (1990) found that an average of 80% of somatic embryos of peanut produced shoots, while an average of 61% produced roots. In soyabean, although 2,4-D produced a larger number of somatic embryos at the induction stage, NAA favoured subsequent conversion into plants (Barwale et al, 1986; Lazzeri et al, 1987). In early studies, the maturation of conifer somatic embryos was attempted by culturing immature embryos on medium containing reduced or zero levels of plant growth regulators (Hakman et al, 1985; Lu and Thorpe, 1987), but maturation was infrequent and the few recovered plantlets were abnormal. ABA has been shown to influence the formation of aberrant forms of somatic embryos (Vasil and Vasil, 1981; Ammirato, 1987) and it was suggested that concentrations below about 8-12μM were beneficial (Becwar et al, 1987; Dunstan et al, 1988). Other hormones such as GA_3 have also been used as an aid to somatic embryo maturation (Ammirato, 1983); for example, application of GA_3 was required for root and shoot development during germination of somatic embryos in *Citrus sinensis* (Kochba et al, 1972).

There has been little published work concerning the recovery of plants from somatic embryos of sweet potato. Chee et al (1990) described plant formation from somatic embryos of sweet potato in response to BAP, NAA and sucrose, showing that a reduction in the sucrose concentration in the basal medium from 3 to 1.6% increased the frequency of plant development form 15 to 32%. Development pathways of somatic embryos and plant regeneration in sweet potato have also been described (Chee and Cantliffe, 1988a). However, these studies were limited to only a few genotypes with much of the published information confined to cv. 'White Star'. Al-Mazrooei (1996) described embryogenic tissues of the genotypes Nemamete, TIB10 and papota that were cultured on MS supplemented with 0.09M sucrose and after eight weeks, following transfer to auxin free

medium, mature embryos (90-100%) conversion from immature embryos and plantlets were recovered at 15 to 50%.

1.3 CRYOPRESERVATION

Cryopreservation techniques are used for the storage of biological material at the temperature of liquid nitrogen (-196°C). The process is becoming an increasingly important aspect of plant biotechnology, since in the future, it will be necessary to establish safe repositories for patented cultures of commercial interest. Cryopreservation can also alleviate some of the problems associated with genetic instability in long-term cultures (Benson and Withers, 1987). It has also been considered a useful system for the long-term storage of vegetative germplasm in the form of isolated meristems. However its use, especially for such organized material, is still not routine, mainly because of the difficulties in preserving the structural integrity (Withers, 1991). The technique is therefore rather more readily applied to the relatively uniform cell populations formed within suspension cultures and as a result successful protocols have been devised for such cultures from a diverse range of species (Withers, 1985).

To maintain germplasm of sweet potato by conventional methods, the tubers or wine-cuttings have to be grown and multiplied in nurseries every year, and clones of sweet potato are being maintained at the International Potato Centre (CIP), Lima and other genebanks by these methods. Such a process is not only time consuming, labour-intensive and expensive, but the material is exposed to the hazards of pests, diseases and various abiotic factors and this may result in the loss of germplasm. Thus, the problem of conservation of genetic resources of vegetatively-propagated methods of storage and cryopreservation has been suggested as one of the approaches.

Cryopreservation techniques were first used successfully with plant cultures when Quatrano (1968) froze suspension culture cells of flax (Linum usitatissimum) to a temperature of -50°C and maintained viability at a level of 14%. This finding was an important initial contribution towards the development of cryopreservation of techniques, although it is apparent that –50°C is a not sufficiently low temperature for long-term storage because of the risk of continued ice-crystal growth (Meryman and Williams, 1982). In later studies, a rapid freezing method was employed to cryopreserve shoot-tips of a few species such as carnation (Siebert, 1976), potato (Bajaj 1977; Grout and Henshaw, 1978, 1980) and cassava (Bajaj, 1977(a)). Later, successful cryopreservation of meristems of diverse species by two-step freezing such as pea, strawberry, carnation, potato, cassava has been reviewed by Kartha (1985). More recently, alginate-coated apical meristems from in-vitro grown wasabi (Wasabi japonica) were successfully cryopreserved following dehydration by vitrification solutions (Matsumoto et al, 1995).

1.3.1 CRYOPRESERVATION PROCEDURE

The cryopreservation procedure can include up to eight steps: preliminary culture (pre-culture), the application of cryoprotectants, encapsulation, evaporative dehydration, freezing, storage, thawing and recovery growth.

1.3.1.1. Preliminary culture

During the period of days, or at least hours, prior to the cryopreservation sequence, most cultures require special manipulation and/or modified culture conditions in order to produce a specimen with maximum freeze-tolerance. The requirements vary according to the culture system and with species; for example with embryos, preliminary culture conditions generally differ for zygotic and somatic embryos (Engelmann, 1992). Somatic embryos are frequently cultured on a high sucrose medium (Engelmann et al, 1995). The

effects of the preliminary culture conditions are probably complex but, in general, there is a reduction in cell size and/or a reduction in the water contents of cells, and equilibration with the cryoprotectants, which are frequently introduced gradually.

1.3.1.2. Choice and application of cryoprotectants

Various interpretations have been given with regard to the mechanism of action cryoprotectants, and a unified hypothesis remains elusive. Indeed, the likelihood is that there are several possible mechanisms which come into play according to particular circumstances. Some agents for example, probably provide protection by reducing the concentrations of intracellular salts at equilibrium (Lovelock, 1953a), particularly in the dehydrated cells, and they may influence the formation of ice-crystals. Depending upon the nature of the cryoprotectants, they could prevent the immediate freezing injury or storage injury or both. In microrganisms, it has been suggested by Mazur (1966) that storage death may result from long-term exposure to residual concentrated solutions and that a cryoprotectants could reduce decay rate either by acting as an innocuous dilutant of toxic solutes or by ameliorating their deleterious action. On the other hand, Rowe (1966) suggested that cryoprotectants interact directly with the cell membranes to stabilize the tertiary structures of the water-lipid-protein complex which appears to be damaged under freezing conditions. In addition, the cryoprotectant may serve to decrease the rate of diffusion of the water molecules to the developing ice-crystals within the cells. Also, Lovelok (1953b) stated that the basic principle of colligative cryoprotectant remains essentially unchallenged and any solute can function as a colligative cryoprotectant provided that it satisfies two requirements: Firstly, it must reach the interior of the cell, otherwise, it will cause osmotic dehydration and produce the very injury it is designed to prevent. Secondly, it must be nontoxic in concentrations sufficiently high to produce a significant influence on the freezing characteristics of the water within the cell. Any

solute or combination of solutes which satisfies these conditions will depress the temperatures of heterogeneous and homogeneous ice-formation and raise the vitrification temperature with the result that at a sufficiently high concentration vitrification should occur during cooling before ice-crystals can be formed (Meryman, and Williams, 1980; see Fig.2).

Cryoprotectants can be grouped into two classes: those that penetrate the cells and those that remain outside the cells. The distinction between the two classes, however, is not clean-cut because lower molecular weight compounds penetrate cells slowly. The rate often depends on the conditions and on the species and in their case, it is difficult to say without further investigation whether the cryoprotectant actions occur predominantly within or outside the cells. In fact, it is quite possible that they have multiple actions, depending on the manner in which they are administered to the cells. The penetrating additives are generally considered to include DMSO, glycerol and methanol, of which DMSO and glycerol have been used most frequently with higher plant tissues. The additives which do not seem to penetrate the cells include mannitol and polyvinyl pyrollidone (PVP), and additives which may or may not penetrate depending on conditions include sucrose and amino acids.

The following cryoprotectants were employed for the present studies:

i) Glycerol

Glycerol which is generally considered to penetrate the cells, can probably act as a colligative agent influencing ice-formation and also by reducing the concentration of other solutes to less damaging levels. Exploitation of this form of cryoprotection depends on the practicality of introducing and removing high concentrations of cryoprotectant. Among the various compounds of this type available for cryoprotection, glycerol appears

to occupy a special position because of its relative lack of toxicity at high concentration. Of all the solutes that have been proposed as cryoprotectants, glycerol is the most water-like in its ability to maintain the hydrophobic forces that are essential to the tertiary and quaternary conformation of macromolecules and stability of membrane bilayers (Tanford, 1973).

Towill and Mazur (1976), however reported that glycerol is either non-penetrating or slow penetrating with tissue culture cells of *Acer* and *Haplopappus*. This was confirmed by freeze-fracture studies in which a reduction in the size of intracellular ice-crystals was only observed following incubation of plant tissue in glycerol for several days before freezing (Fineran, 1970; Robards and Parish, 1970). In contrast, glycerol has been reported to penetrate suspension culture cells of carrot (Nag and Street, 1975). The cytotoxicity of penetrating additives is a function of temperature and the period of exposure. At low temperatures, glycerol is less damaging but this may only be a consequence of the temperature coefficient of cellular permeability (Mazur et al, 1974).

ii) Dimethylsulphoxide (DMSO)

The mechanism of action of DMSO as a cryoprotectant is at present not fully understood. It may be considered that there are at least three locations where DMSO may act to avoid freezing injury, i.e. in the exterior solution, on the membranes and in the interior solution (Sakai, 1985). DMSO has proved to be an excellent cryoprotectant and it has been extensively used with both animal and plant cells. DMSO penetrates the cell rapidly but it is potentially cytotoxic and it inhibits cellular metabolism at a low and high concentration. At higher concentrations, it reduces respiration and inhibits RNA and protein synthesis in isolated plant cells and tissues. It is, however reported that DMSO is

easily washed out from the cells once the cryopreservation procedure is completed (Bajaj et al, 1970 a; Towill and Mazur; 1976; Morris and Canning, 1978).

iii) Proline

Proline has been shown to act as an effective cryoprotectant with corn suspension cells (Withers and King, 1979). However, no survival was in the frozen carnation shoot apices in the presence of 10% L. proline plus 5% sucrose even following pre-culturing on agar medium containing proline (Uemura, 1981).

iv) Sucrose

It has been shown that sucrose plays a very important role in the acquisition of resistance to desiccation by seeds of *Glycine max*, *Pisum sativum* and *Zea mays* (Koster and Leopold, 1988). Crow et al (1984) found that sucrose can maintain the liquid crystalline state of the membrane bilayer and can stabilise proteins under extreme dry conditions. thus protecting the structural integrity of the membranes by preventing membrane fusion, phase transition and phase separation.). It has been suggested that these properties are relevant to cryopreservation (Kendal et al, 1983).

Sucrose also reduces the moisture contents of cells slowly (Uragami, 1991) due to osmotic action and, as a result of its gradual uptake, it decreases the freezing point and the amount of freezable water present in the cells. A considerable amount of sucrose uptake and its subsequent partial dissociation into glucose and fructose during pre-culture in the presence of high sucrose levels was demonstrated with axillary buds of asparagus (Uragami et al, 1990). This dissociation inside the cell causes a considerable increase in osmolarity since a 1M solution dissociated sucrose releases a 2M concentration of the monosaccharides glucose and fructose. An indirect effect of sucrose during prolonged pre-culture periods could be the accumulation of endogeneous compounds induced by the

mild osmotic stress, that then offer protection against further water stress and cryopreservation. Proline is such a compound that was found to accumulate in immature embryos of *Zea mays* during pre-culture in the presence of 15% and 20% (w/v) sucrose (Delvallee et al, 1989).

A limited number of more recent reports describe successful cryopreservation by means of a sucrose pre-treatment, followed by direct transfer to liquid nitrogen without any additional cryoprotection. They involved somatic embryos of oil-palm (*Elacis guineesis*) (Engelmann and Dereuddre, 1988), and axillary buds of asparagus (Uragami, 1993). A combination of two treaments (high sucrose and evaporative dehydration) proved successful in the case of coconut embryos (Assy-Bath and Engelmann, 1992). In a recent report on cryopreservation of banana meristem cultures (Panis et al, 1996), the proliferating meristems were pre-cultured for 2-4 hrs. on MS medium supplemented with sucrose levels ranging from 0.1M o 0.75M and then plunged directly into liquid nitrogen (-196°C). The optimal sucrose concentration of 0.4M and 0.5M gave survival of between 12 and 72% depending on the cultivar.

v) Mannitol

Mannitol is essentially non-penetrating and it most probably acts as an osmotic agent. Supplementation of the culture medium with an osmotically active compound such as mannitol, sucrose etc has been found to be effective in increasing the freezing resistance. Mannitol at concentrations of 3.3 and 5.2% (w/v) has been found to be beneficial in reducing the mean cell volumes of cells of *Acer pseudoplatanus* and *Capsicum annuum* and increasing their post-freezing (Withers and Street, 1977a). However, pre-culture on a medium supplemented with 5.2% mannitol did not enhance the survival of *Nicotiana sylvestris* cells subjected to a cryopreservation procedure (Maddox et al, 1983). The

osmotically active compounds such as mannitol therefore possess cryoprotective properties, although the exact mechanism of action of mannitol is unclear. Pritchard et al, (1986) showed that sycomore cells, grown to exponential phase in either mannitol or sorbitol supplemented media exhibited increased levels of total and soluble protein and respiratory activity. A decreased amount of free proline in association with enhanced levels of survival.

1.3.1.3 Encapsulation

Encapsulation is a technique that was originally developed for the protection of somatic embryos as part of the synthetic seed procedure. It involves the coating of somatic embryos in a protective gel which is sufficiently durable to allow handling and storage whilst allowing the embryos to germinate under suitable conditions. The encapsulation of somatic embryos or meristems in beads of calcium alginate also apparently increases the resistance to dehydration and to the low-temperature used in conventional cryopreservation techniques. Encapsulation techniques using hydrogels such as calcium alginate and somatic embryos of various angiosperm species reviewed by Redenbaugh et al, (1987) (see section 1.2.3.1). New cryopreservation techniques involving encapsulation/dehydration were developed for meristems and somatic embryos (Dereuddre et al, 1990). Somatic embryos of *Coffea canephora* were successfully cryopreserved after being exposed to a series of preparative steps: (I) somatic embryos were progressively pre-cultured on media with increasing concentrations of sucrose (0.3, 0.5, 0.7 and 0.8M), allowing 3-4 days on each medium; (ii) they were then encapsulated in alginate beads that contained 0.5M sucrose; (iii) these beads were dehydrated to 13% moisture content (F.Wt. basis) and plunged into liquid nitrogen. After this treatment 63% of cryopreserved embryos remained alive and 50% of them grew directly without formation of secondary embryos and callus (Hatanaka et al, 1994).

1.3.1.4 Evaporative dehydration

Evaporative dehydration further reduces the amount of water available for ice-formation. If this process is carried out in combination with sucrose pre-treatment, cellular solute concentrations may be high enough to promote vitrification (see section 1.3.1.5). It has been shown with several species that the combination of encapsulation and high sucrose in the pre-treatment and evaporative dehydration of somatic embryos, can enhance the survival (Plesis et al, 1993; Blakesley et al, 1995).

1.3.1.5 Freezing

Water that is cooled to sub-zero temperatures will remain in a supercooled liquid state until an ice-nucleation event occurs, generally in the regions of a non-aqueous surface such as the container wall, a dust particle or a large molecule. The effectiveness of these nuclei varies according to the size and the result is that a heterogeneous array of ice-crystals is formed. If the heterogeneous ice-crystallisation event fails to occur supercooling continues until the water becomes self-nucleating in the regions of $-40^{\circ}C$, and a more homogeneous array of smaller ice-crystals is formed. Under special circumstances, generally involving ultra rapid cooling to the temperatures below $-100^{\circ}C$, water changes directly from a liquid state to a non-crystalline glassy or vitrified state. The exact temperatures at which these events occur varies with the purity of the water and solutes The temperatures of heterogenous and homogeneous nucleation are both lowered and conversely the vitrification temperature is raised by an amount dependant on the concentration (see Fig 2.). . When these events occur within cells, it is the large ice-crystals formed by heterogeneous nucleation that have the greatest potential to cause damage. Successful cryopreservation strategies generally seek to reduce the amount of freezable water within a cell and to control the routine of any nucleation event that might take place.

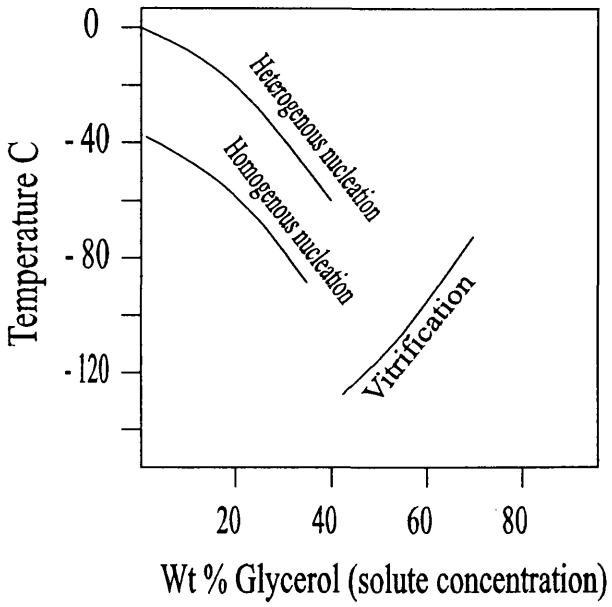


Fig. 2. Mechanism of freezing (Meryman and Williams,1985)

1.3.1.5.1 Two-step cooling procedures

Two-step cooling procedures generally involve an initial slow-cooling stage during which water leaves the cells as a result of osmotic dehydration. The nucleation or vitrification event then occurs during the second stage when the cells are transferred directly to liquid nitrogen for rapid cooling.

During the first stage of cooling extra cellular ice formation either occurs spontaneously or is induced and the ice invades the cell but not the plasma membrane. As the ice develops, the extracellular solution becomes more concentrated, and it is generally argued that the plasma membrane acts as a barrier that prevents the ice from seeding the cytoplasm, so that the cytoplasm remains supercooled. The resulting increase in the osmolarity of the external solution causes water to leave the cell, thus reducing cell volume and concentrating the cell contents. The rate of cooling during the first stage is likely to be critical since too fast a rate might not allow sufficient water to leave the cells

before intra-cellular freezing occurs, and too slow a rate might result in over dehydration and destruction of membranes.

There are different procedures for achieving this state of osmotic or protective dehydration, which include slow-cooling at either constant or varying cooling rates, or holding samples at one or more intermediate sub-zero temperatures.

The success of the two-step freezing procedures depends upon a number of additional factors such as the pre-treatments and cryoprotectant type, the physiological state of the material and the temperature prior to immersion in liquid nitrogen. The most common method for cryopreservation of plant cells involves initial slow cooling at a constant rate in the range 0.5 to 2 $^{o}Cmin^{-1}$ to a terminal temperature between –30 and –40°C, followed by transfer to liquid nitrogen (Withers, 1985). The two-step freezing method was first described by Luyet and Keane (1955) and subsequently applied to a number of cell types (Farrant et al, 1977). The successful applications of this procedure with shoot meristems of a range of plant species, including meristems of pea, strawberry, potato, cassava, carnation are listed by Kartha (1985).

1.3.1.5.2 Rapid cooling procedures

Rapid cooling is accomplished by the direct immersion of the cryoprotectant-treated specimens in liquid nitrogen. The cooling rate is in the order of several hundred degrees per minute, and at such rapid cooling rates, the intracellular fluids do not have sufficient time to equilibrate with the external ice before intracellular ice formation takes place. Such ice formation may be lethal, but it is also possible that ultra-rapid cooling may prevent the growth of intracellular ice-crystals by rapidly passing the cells through the temperature zone in which lethal ice-crystals grow. Alternatively, depending on solute concentrations, vitrification may occur. Rapid cooling has the advantage of technical

simplicity and controlled-cooling equipment is not required, but they have the possible disadvantage that there is little scope for fine adjustment of the cooling rates to suit the requirements of particular species or genotypes (Henshaw, 1987).

1.3.1.6 Storage

At temperatures higher that approximately –100°C, ice-recrystallisation can occur as a result of the transfer of water molecules from small to larger ice-crystals. Since this process can produce potentially lethal ice-crystals, it is important to store frozen cultures at sufficiently low-temperatures and the temperature of liquid nitrogen is the most convenient from the practical point of view. Samples are maintained in an insulated storage vessel containing liquid nitrogen, the levels of which are regularly monitored and replenished as required.

1.3.1.7 Thawing

The temperature and rate at which tissues are thawed, is dependent on the freezing method. Thawing is critical in vitrified tissues or in tissues containing micro-crystals of ice. Since ice-recrystallization can occur during the warming periods, once the temperature rises above approximately –100°C. In conventional cryopreservation methods, thawing is actually achieved as rapidly as possible by plunging the cryovials into water maintained at 40°C and, once all the ice has disappeared, the samples are transferred to a more normal incubation temperature. Thawing rates for tissues that have been frozen by encapsulation and/or dehydration can be less critical if the water content is so low that ice-recrystallization is unlikely to occur.

1.3.1.8 Post-thaw recovery

Although there are colorimetric and other chemical methods by which the viability cells can be evaluated (Finkle and Ulrich, 1979), post-thaw recovery growth is the ultimate measure of survival. Henshaw et al (1980) suggested that recovery growth is usually

carried out on a semi-solid medium similar in nutrient composition to the standard medium. This was possibly supplemented by activated charcoal to absorb toxins released by lethally damaged cells or hormones to stimulate the desired pattern of growth. Withers (1980) suggested that the composition of the post-thaw culture medium plays a significant role in post-thaw survival. In the case of an organized structure, for example, survival is largely dependent upon the hormone composition of necessary medium and, if this medium contains high concentrations of auxin and cytokinin to stimulate proliferation. Plants may regenerate via intermediate callus formation and adventitious shooting so that a high recovery rate might be at the expense of regenerant quality. Withers (1985) has identified aspects of the post-thaw phase that may affect recovery. Post-thaw washing has been considered important because it removes potentially toxic cryoprotectants, but there is also possibility that it aids the removal of important metabolites from already damaged and leaky cells. This probably accounts for the deleterious effects of washing described by Withers and King (1979).

The environmental conditions during the post-thaw period may also be important. Light has been shown to enhance the damaging effect of low-temperature injury and this is probably mediated by free-radicals, particularly in physiologically disorganised green tissues such as shoot meristem-tips (Benson, 1990). Thus it may be beneficial to allow green organised structures such as meristems to recover for a short period in the dark to prevent or decrease photo-oxidation.

1.3.2 CRYOPRESERVATION OF SOMATIC EMBRYOS

The potential importance of somatic embryogenesis for propagation, genetic manipulation and genetic conservation has stimulated the development of protocols for

cryopreservation of somatic embryos or embryogenic tissue. Whilst it is well established that zygotic embryos in 'orthodox seeds' with low water contents are relatively amenable to cryopreservation techniques (Stanwood et al, 1986), less is known about cryopreservation of somatic embryos since work has been confined to a restricted number of species.

In the earlier studies with somatic embryos of carrot (Withers, 1979) and citrus species (Marin and Duran-Vila, 1988) the conventional two-step freezing procedure combination with low concentrations of cryoprotectants was successfully employed. More recently, however, there has been reports of success with the alternative methods involving high sucrose pre-treatments combined with encapsulation and or evaporative dehydration (see section 1.3.1.2 and 1.3.1.3) or with 'vitrification' mixtures of other cryoprotectants (see section 1.3.1.5). A combination of two treatments (high sucrose and evaporation dehydration) together with two-step freezing proved successfully in the case of coconut embryos (Assy-Bath and Engelmann, 1992). Dumet et al (1993) stated that somatic embryos of seven different clones of oil palm were successfully cryopreserved by a process involving treatment with high concentrations of sucrose (0.75M) for seven days, followed by partial dehydration (16 hours with silica-gel before freezing). Tessereau et al (1994) successfully cryopreserved somatic embryos of two species of coffee (*Coffee canephora*, P) and carrot (*Daucus carota*, L) by a two-step protocol involving cooling to at least –20°C prior to immersion in liquid nitrogen. Prior to freezing, the somatic embryos of carrot were incubated for 21 hours in basal medium containing 0.15M to 0.4M sucrose and the coffee somatic embryos were subjected to a hardening treatment involving a high concentration of sucrose 0.4M and 1μM ABA. De-Boucaud et al (1994) successfully cryopreserved somatic embryos of walnut (*Juglans regia*) by a two-step and

rapid freezing protocol involving cooling at –40°C prior to immersion in liquid nitrogen. Prior to freezing, the somatic embryos (1-2mm) were precultured on DKW (Driver and Kuniyuki) basal medium supplemented with 5% DMSO and 0.5% proline, DKW medium concentrations of sucrose (0.25M to 1.0M) and evaporative dehydration in a laminar flow hood. In this case thermal analysis performed on the somatic embryos, both naked and encapsulated in calcium arginate beads showed the dehydration times (1.5 and 3 hrs. respectively) that were necessary to achieve vitrification on cooling. Nishizawa et al (1993) successfully cryopreserved the embryogenic cells of asparagus (*Asparagus officinalis*, L) by vitrification. Prior to freezing, the cells were cryoprotected with a mixture of 2M glycerol and 0.4M sucrose and dehydrated with a new vitrification solution (designated PVS3) at 0°C for 20 minutes prior to plunging into liquid nitrogen. PVS3 contained 50% (w/v) glycerol and 50% (w/v) sucrose in water. The vitrified cells in LN_2 were rapidly thawed in a water bath at 40°C and then expelled into LS medium supplemented with 1,2M sucrose. The survival of cells treated with PVS3 was 80%.

1.3.3 CRYOPRESERVATION OF SHOOT MERISTEM-TIPS

Cryopreservation of shoot meristem-tips is potentially one of the most important in-vitro techniques for the long-term storage of vegetatively-propagated species such as sweet potato. This is because maintenance costs and the chances of genetic instability are likely to be reduced in comparison with continuously growing cultures (W.M. Roca, person. comm.). Moreover, since meristematic cells are small and densely cytoplasmic with low water contents, they might seem to be particularly suitable for the application of cryopreservation techniques. However, in comparison with cell suspension cultures, there are the additional complications of their relative bulk, compared with the cell unit within a suspension culture, and the maintenance of organization. Nevertheless, Seibert (1976),

Grout and Henshaw (1978) reported the successful cryopreservation of carnation and potato mersitem-tips, using ultra-rapid freezing methods by which the tips were directly immersed in liquid nitrogen.

Considerable success has been achieved in recent years with the further development of cryopreservation procedure for shoot mersitem-tips, particularly with two-step protocols (Withers and Williams, 1980; Kartha, 1985). However, many problems are still encountered and so far the techniques have only limited application for routine germplasm storage. In particular, there can be widely differing responses to freezing among different species and even between different genotypes from the same species. With sweet potato mersitem-tips, for example, preliminary studies at Bath with two-step and rapid freezing protocols which had already been used successfully with a number of the species, proved to be completely unsuccessful (deGoes, 1993; D. Blakesley, person. Comm.). Since this preliminary work was carried out, however, various alternative cryopreservation procedures have been described, including those involving pre-treat ments with high concentrations of sucrose combined with encapsulation and/or evaporative dehydration (see section 1.3.1:1-3) or with vitrification including a mixture of cryoprotectants (see section 1.3.1.5). Paulet et al (1993) successfully cryopreserved apices from in-vitro plantlets of sugarcane by using an encapsulation-dehydration technique in which the encapsulated apices were pre-cultured for two days in liquid medium supplemented with 250g^{-1} sucrose and then dehydrated for 6 hours under a laminar flow hood followed by rapid freezing. Survival after freezing in liquid nitrogen ranged between 38% and 91% for five varieties. Hirata et al (1995) successfully cryopreserved shoot primordia of (Armoracia rusticana) horse radish using the encapsulation-dehydration technique. The shoots were first encapsulated in alginate

medium supplemented with 0.5M sucrose and precultured on MS medium supplemented with 0.5M sucrose dehydrated to 24% moisture content before transfer into liquid nitrogen; the survival rate of shoot primordia was 46%. Na and Kondo (1996) successfully cryopreserved shoot primordia of Vanda pumila by rapid freezing. The shoot primordia were precultured on B5 liquid medium supplemented with 1.0mg/l ABA and dehydrated under 40-45% RH down to 24% relative water content prior to plunging into liquid nitrogen; the survival of shoot primordia was 65%. Brison et al (1995) stated that shoot-tips of two interspecific *Prunus* rootstock were successfully cryopreserved using a two-step freezing method. Shoot-tips from hardened plantlets were precultured for 24 hrs. on MS medium supplemented with 5% DMSO and 2% proline and transferred to PVS2 solution for 20-40 minutes. Samples were then cooled at 1°C/min to –40°C prior to plunging into liquid nitrogen. Shoot-tips were rinsed with MS medium containing 1.2M sucrose. The survival of shoot-tips was 69% and 74%.

Towill and Jerret (1992) successfully cryopreserved shoot-tips of sweet potato (*Ipomoea batatas*, L) by vitrification. Shoot-tips were exposed to 10, 20 and 40% PVS2 at 22°C and 60, 80 and 100% PVS2 (30% glycerol, 15% DMSO and 15% ethylene glycol) at 0°C for 60, 20, 10, 5 and 2 minutes prior to plunging into liquid nitrogen. The survival of shoot-tips was 26%. Matsumoto et al (1995) successfully cryopreserved encapsulated apical meristem of *Wasabia japonica* by a process involving vitrification; apical meristem were cultured on MS medium containing 0.3M sucrose at 20°C for one day before trapping into alginate coated beads containing a mixture of 2M glycerol plus 0.4M sucrose. The encapsulated meristems were treated with PVS2 solution for 30 minutes at 25°C or 70-100 minutes at 0°C prior to transfer to liquid nitrogen. The survival of encapsulated vitrified meristems was 95%. Matsumoto et al (1995) successfully cryopreserved apical

meristems of Japonica Pink lily by vitrification. The apical meristems were precultured on MS medium containing 0.3M sucrose for one day and then loaded in a mixture of 2M glycerol plus 0.4M sucrose for 20 minutes. The treated samples were treated with PVS2 (30% glycerol, 15% DMSO, 15% ethylene glycol) solution for 20 minutes prior to plunging into liquid nitrogen. The survival of apical meristem was 80%.

1.4 AIMS OF PROJECT

The main objective of the project was to develop improved techniques for the in-vitro conservation of sweet potato germplasm based on cryopreservation procedures. Earlier work at Bath (de Goes, 1993; D. Blakesley, Person. Comm.) had already shown that shoot meristem of sweet potato are recalcitrant with regard to the earlier developed cryopreservation techniques, involving either rapid or two-step freezing protocols in combination with low concentration of cryoprotectants. The intention, therefore, was to investigate the effectiveness of the more recently developed techniques involving the much higher concentrations of cryoprotectants in combination with encapsulation and/ or evaporative dehydration. These are believed to encourage vitrification rather than ice-crystal formation during the freezing process (see section 1.3.1.5). These techniques were investigated both with embryogenic tissue and with shoot meristems.

It was recognised that a cryopreservation procedure based on the use of shoot meristem-tips would be required for germplasm storage purposes because of the need for adequate levels of genetic stability during the *invitro* growth periods before and after cryostorage. Following difficulties encountered with shoot meristem tips in preliminary studies, confirming the problem described by de Goes (Loc. Cit.) and Blakesley (Person. Comm), it was decided to focus attention initially on the cryopreservation of

embryogenic tissues. This decision was taken primarily for practical reasons, because it was believed that the large amount of replicated tissue that would be required in the large-scale experiments could be more readily produced. In addition however, it was considered that there is now a need to develop cryopreservation procedures for the embryogenic tissue itself because of its importance for the development of transformation protocols and possibly for mass propagation. For both of these purposes, the availability of an efficient cryostorage procedure for embryogenic tissue from a wide range of genotypes would avoid the problems of morphogenesis and genetic instability that might be otherwise associated with long-term maintenance. With this approach, therefore, it was first necessary to establish protocols for the efficient production of embryogenic tissue from a diverse range of sweet potato genotypes. Then, after the cryopreservation studies with the embryogenic tissue, the intent was to attempt the adaption of any successfully protocols for use with the previously recalcitrant shoot meristem-tips.

2. MATERIAL AND METHODS

2.0 MATERIAL AND METHODS

2.1 PLANT MATERIAL

The sweet potato genotypes listed, together with their origins in table 2.1.. were maintained as shoot culture under the conditions described in section 2.3.

2.2 MEDIA PREPARATION

Unless otherwise stated, the basal medium used was that of Murashige and Skoog (1962) made up from reagents supplied by Imperial Labs, Andover, U.K. and stored at 4°C. The media were supplemented with 2% (w/v) sucrose and plant growth regulators (PGR's) and were made up to volume with double-distilled water. Where appropriate PGR's were added to the medium as indicated in the Results section 3 prior to adjustment of the pH to 5.7 with 0.1M NaOH or 0.1M HCl , Agar (Oxoid No.3, Unipath, UK) was added at concentration of 7.0 g/l unless otherwise stated and media were sterilised by autoclaving at 120°C and 1.4bar for 15 minutes in 500ml or 1 litre Schott Duram glass bottles. Media were allowed to cool to 45°C before dispensing at 25ml per 9 cm single vented petri dish (Sterling Ltd, UK). The dishes were stored until use (no more than two weeks) in plastic bags in darkness at room temperature.

For cultures grown in glass jars with plastic screw caps, agar was added to liquid medium and melted by boiling in a microwave oven. The medium was dispensed at a volume of 30ml per 175ml screw capped powder jar and as described above.

2.3 SHOOT CULTURE MAINTENANCE

Stock shoot cultures from the 15 genotypes of sweet potato described in Section 2.1 were routinely maintained in 175ml glass jars. At subculturing, the leaves were removed with a sterile scalpel blade (Swann Morten No.11) and the shoot-tip consisting of three axillary buds was placed on 30ml basal medium (MS) with 2% (w/v) sucrose. The remaining

plant stems were cut into pieces, each containing three axillary buds. The stem pieces were transformed to the glass jars (two pieces per jar) which were then incubated for eight weeks.

2.4 CULTURE INCUBATION CONDITIONS

All cultures were incubated at 25±1°C with a 16hr. photoperiod (70μM $m^{-2}s^{-1}$ PAR) from warm white fluorescent tubes unless otherwise stated.

2.5 SOMATIC EMBRYOGENESIS

Embryogenic tissue cultures were established from axillary meristem-tips (0.2-1.0mm in length) excised from eight genotypes of actively growing shoot cultures (Results section 3.1). The meristem-tips were placed on MS_2 medium containing one of the auxin 2,4-D, 2,4,5-T and Picloram (Sigma Co. Ltd., UK) as indicated in the Results section (3.2 to 3.5) and incubated for six weeks.

For routine maintenance, all embryogenic tissues were then subcultured every four weeks onto fresh MS_2 medium containing 5μM 2,4-D.

Embryogenic tissue with the appropriate morphological characteristics (shiny with globular or torpedo-stage embryos) and colour (red or yellow), according to the genotypes (see table 2.2) was subcultured and the remaining non-embryogenic tissue was discarded. The embryogenic competence of each genotype was at each sub-culture by incubating samples on basal MS_2 medium and examining for embryo formation after four weeks.

For routine maturation and germination of embryos, clumps of embryogenic tissue (2.0 – 4.0mm) were transferred to semi-solid MS_2 medium supplemented with 1μM GA_3 for one to two weeks prior to transfer to MS_2 medium supplemented with 0.06M sucrose (MS_2).

2.6 CRYOPRESERVATION

2.6.1 PRE-CULTURE TREATMENT

The high sucrose concentrations (up to 1.0M) which were investigated for possible cryoprotectant qualities with embryogenic tissue could only be introduced progressively over periods of several days. These pre-treatments involved the incubation of the tissue in a series of media containing increasing sucrose concentrations up to a maximum of 1.0M over periods of up to 10 days. The details of sucrose concentrations and periods of incubation in the various stages are provided in the descriptions of individual experiments in the Results section (Chapters 4 and 5).

An alternative cryoprotectant mixture (PVS_2) described by Sakai et al (1990) and containing 30% (v/v) glycerol, 15% (v/v) ethylene glycol and 15% (v/v) DMSO was investigated with shoot meristem-tips. The full strength mixture (100% PVS_2) was prepared in MS medium containing 0.4M sucrose and it was used to prepare a series of media containing increasing concentrations of PVS_2 (20, 40, 60, 80 and 100% v/v), each combination with MS medium and 0.4M sucrose. The meristem-tips were incubated under the standard conditions (see section 2.4) in the different PVS_2 media (0.25ml in a test tube) for the periods of time indicated in the individual experiments described in the Results section (Chapter 5).

2.6.2 ENCAPSULATION

For some of the experiments, as indicated in the Results section, embryogenic tissues or meristem-tips were encapsulated in alginate beads prior to the pre-culture treatments. For routine preparation of the alginate beads, embryogenic aggregates (1.0- 2.0 mm in diameter) or meristem-tips were suspended in liquid MS medium supplemented with 3% (w/v) sodium alginate (Sigma Co.Ltd., UK) and 0.1M sucrose. After 20 minutes, the

mixture was dispensed dropwise with 100mM calcium chloride and 0.1M sucrose so that each drop contained one of the aggregates. The resulting calcium alginate beads (4.5 – 5.5 mm in diameter) were removed from the liquid medium after 5 minutes and collected on sterile filter paper (Whatman No.2). The beads were then transferred to a series of semi-solid MS media supplemented with 2,4-D and increasing concentrations of sucrose (0.1, 0.4, 0.7 and 1.0M); for details see section 4.20 and the indicated experiments in the results section (Chapter 4).

2.6.3 Evaporative Dehydration

2.6.3.1 Encapsulated Tissues

The beads containing somatic embryos or shoot meristem-tips were dehydrated by transferring them to an empty open petri dish (ten beads per dish) and exposing them to the sterile air-flow in a laminar flow hood for up to six hours (Plate 1). After dehydration, seven beads from each replicate batch transferred to a 2ml polypropylene cryovial (CamLab. Co. UK) prior to freezing and three beads were transferred to an oven at 105°C for determination of moisture content. The moisture content was calculated according to the following equation:

$$\text{Moisture content (\% Fresh Mass)} = \frac{\text{Fresh weight-Dry weight}}{\text{Fresh weight}} \times 100$$

2.6.3.2 Non-encapsulated tissue

Following transfer through the series of sucrose-containing media (see section 2.6.1), three replicate batches of seven embryogenic aggregates were transferred to a Whatman 2 filter paper carrier (2.0 x 0.5cm) and dried in a closed 9 cm petri dish containing 2.0 g of dried silica-gel for up to five hours (Plate 1). After dehydration, the carriers were transferred to 2ml polypropylene cryovials for freezing. A similar procedure was employed for dehydration of shoot meristem-tips, but for nodal segments a larger amount of silica-gel (15g) was employed in each petri dish. The moisture contents were estimated

on a percentage fresh mass basis by the procedure described in 2.6.3.1 from three replicate batches of embryogenic tissue or meristem-tips

2.6.4 FREEZING

2.6.4.1 TWO– STEP FREEZING

Two-step freezing was carried out as follows:

The first stage, involving slow-cooling of the cryovials from ambient to 0°C at 10°C min^{-1} and then to –40°C at 0.5°C min^{-1} was carried out in a Planer Cryo 10 Series III Programmable freezer (Planer Products, Plc, UK) (Plate 2). This was followed immediately by the second stage in which the cryovials were rapidly frozen by plunging directly into liquid nitrogen (see section 2.6.4.2).

2.6.4.2 Rapid Freezing

Rapid freezing was achieved by plunging the cryovials directly into liquid nitrogen (1-196°C) contained in a 2l Dewar flask. The cryovials remained in the liquid nitrogen for one hour before thawing (Plate 2).

2.6.5 THAWING

Cryovials containing the frozen samples were quickly thawed in a bath at 40°C for one minute. The samples were then removed from the cryovials and transferred to semi-solid MS medium containing various supplements according to the experiment (see Results section, Chapter 4 and 5).

2.6.6 RECOVERY

After thawing, the cultures were incubated at 25±1°C in darkness for 24 hr. (somatic embryos) or 48h (shoot meristem-tips), before transfer to the standard incubation conditions in the light (see section 2.4). The encapsulated samples were removed from beads after the first stage of incubation in the dark for further incubation under the light conditions.

The survival of embryogenic tissues was monitored over subsequent weeks and the final data were collected after four weeks. Survival was recorded on the basis of the proliferation of either embryogenic tissue or non-embryogenic callus. The capacity of this tissue to produce mature embryos capable of germination was assessed using the regeneration procedure described in section 2.5.

2.6.7 Statistical analysis

The statistical significance of the differences between genotypes and treatments were tested using Genstat 5.3. The method used to analyse the number of embryogenic explants from a fixed number of explants was generalized linear modelling with a binomial distribution (McCullagh and Nelder, 1989). In some experiments two-way analysis of variance of arsin transformed data was employed to identify significant interaction between treatment and genotype.

Genotype	Source
865M	**Upland Crops Research Institute, Guangdong-China**
1023M	" " " " " " " " " "
30MT	" " " " " " " " " "
209M	" " " " " " " " " "
207M	" " " " " " " " " "
132M	" " " " " " " " " " (via P. Lepive, Gambloux, Belgium)
TIB10	International Institute for Tropical Agriculture, Nigeria
Papota	Potato Research Institute, US Department of Agriculture, USA
Nemanete (CIP-420005)	International Potato Centre, Lima-Peru
Jewel (CIP-440031)	" " " " " " " "
Imby3120 (CIP-440037)	" " " " " " " "
Sunny (CIP-440040)	" " " " " " " "
715-2498 (CIP-440038)	" " " " " " " " (via C. Newell, Agricultural Genetic Company Ltd, Cambrige, UK)
Brondal	Not known (via G.G. Henshaw, University of bath, UK)
Jersey orange	Not known (via G.G. Henshaw, University of Bath, UK)

Table 2.1 Source of sweet potato germplasm

Genotype	Morphological Characteristics	
	*Degree of friable callus formation	Colour of embryogenic tissue
865M	+	Red/ purple
1023M	+	Red/ purple
TIB10	+	Red/ cream
Papota	+	Red/ yellow
30MT	+	Yellow
209M	+	Yellow
207M	++	Yellow
132M	+++	Pale yellow

- Friable callus (Non-embryogenic calli) scored after four weeks.

+ = 1.0-2.0 mm

++ = 2.0-4.0 mm

+++ = 4.0-6.0 mm

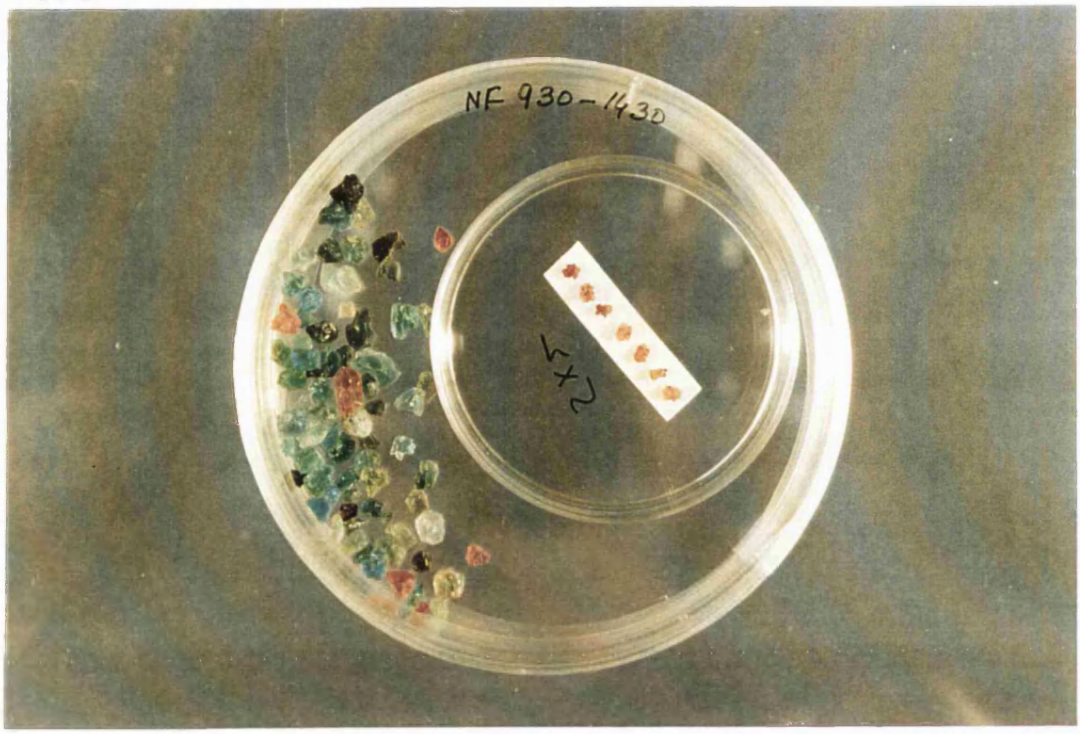
Table 2.2 Morphological characteristics of embryogenic and non-embryogenic callus in eight genotypes of sweet potato

Plate 1: Dehydration process for non-encapsulated and encapsulated embryogenic tissues.

1.A: Embryogenic aggregates placed on filter paper carrier (2.0 x 0.5cm) for drying in a 9cm Petri dish containing 1.8g of silica-gel for up to 5h.

1.B: Encapsulated embryogenic tissues pre-cultured on a series of MS basal media supplemented with 5μM 2,4-D and increasing concentration of sucrose (up to 0.7M) for 8d before transfer to an empty open Petri dish for dehydration in the sterile air flow of a laminar flow hood (up to 5h).

1.A



1.B

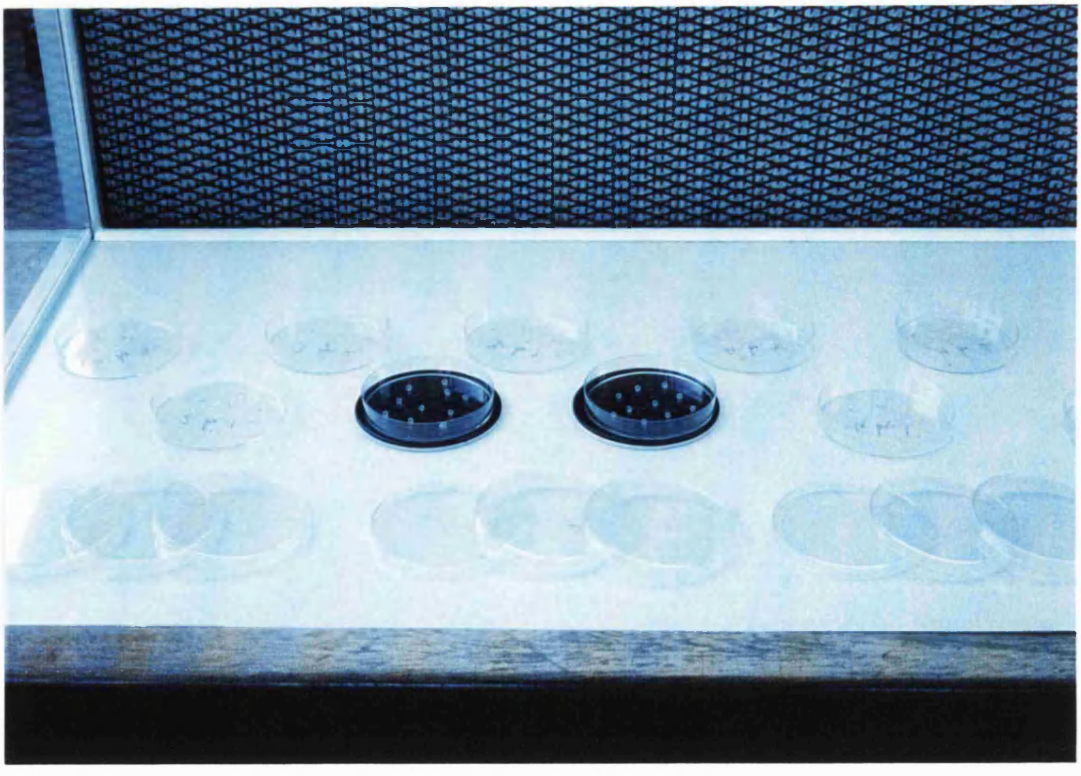
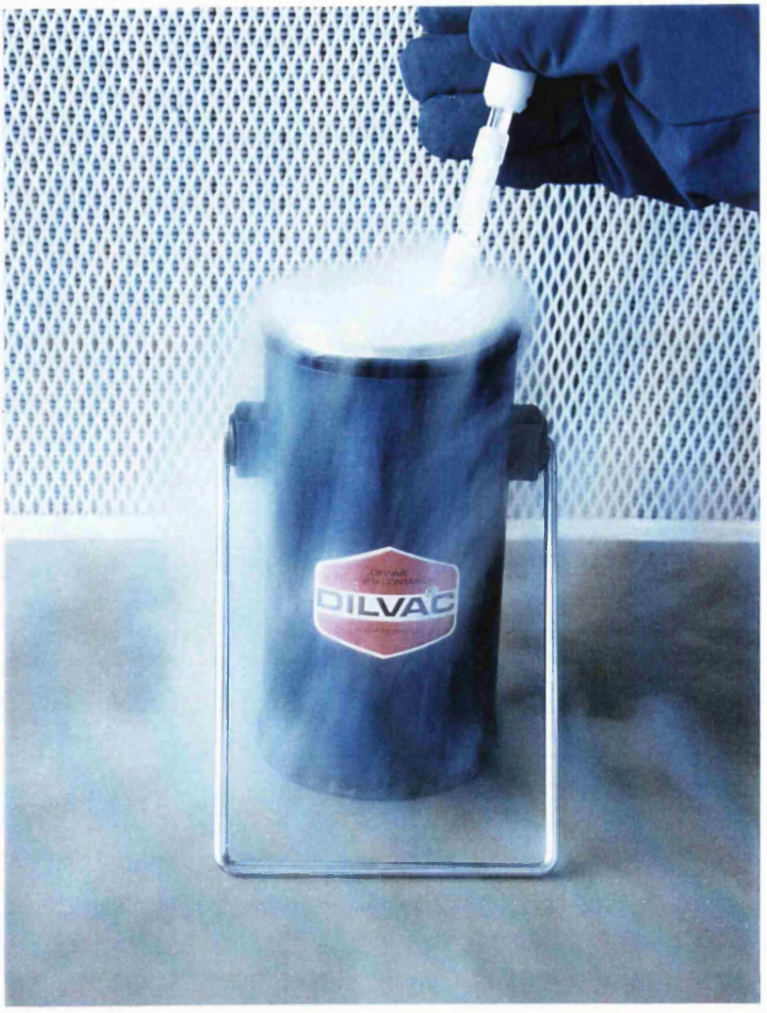


Plate 2: Rapid and two-step freezing processes

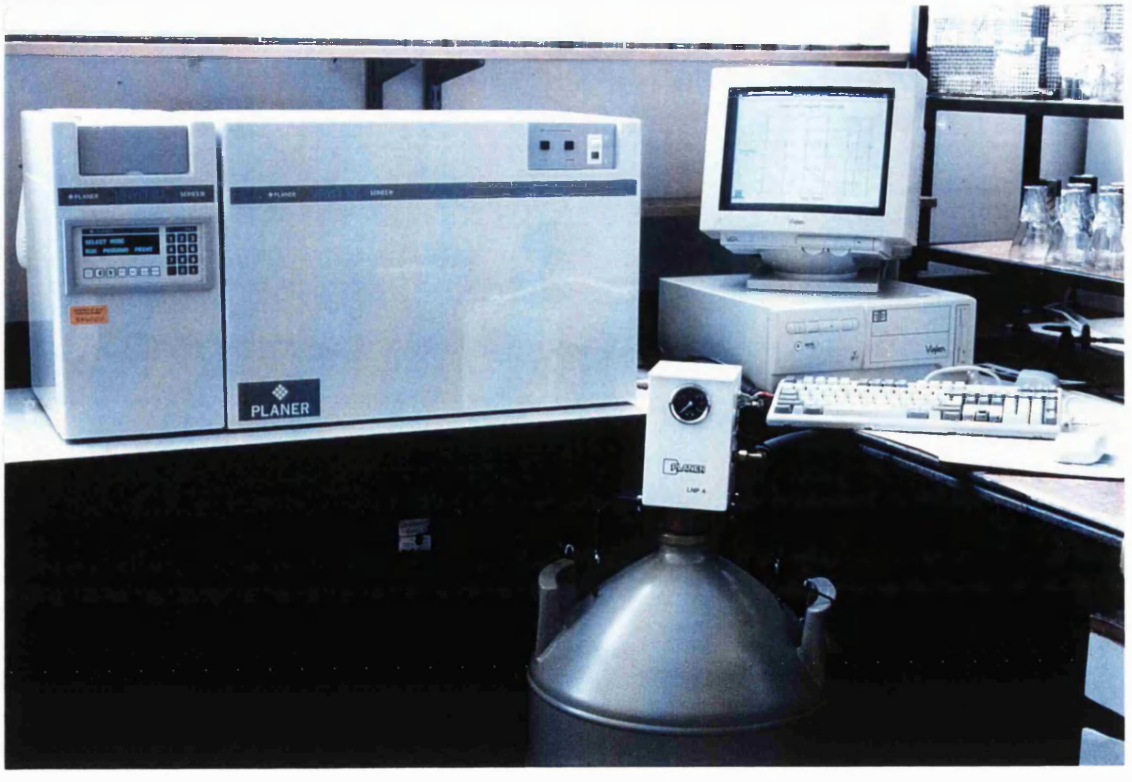
2.A: Rapid-freezing : 2l Dewar flask containing liquid nitrogen (-196°C). The dehydrated beads were transferred to a 2ml polypropylene cryovial which was plunged directly into the liquid nitrogen.

2.B: two-step freezing : The first stage involved slow-cooling from ambient to 0°C at 10°C min-1 and then to –40°C at 0.5°C min-1 in a programmable freezer, followed immediately by the second stage in which the cryovials were plunged directly into liquid nitrogen.

2.A



2.B



3. RESULTS AND DISCUSSION

3. RESULTS AND DISCUSSION

3.0 INDUCTION OF SOMATIC EMBRYOGENESIS

The process of somatic embryogenesis has an increasingly important role to play in crop improvement programmes, by providing a target tissue for the development of genetic transformation techniques. It may also provide a basis for clonal propagation and synthetic seed production in certain crops. There are, however, a number of difficulties associated with the development of suitable embryogenic cultures with the result that the production of high quality embryogenic tissue from a wide range of genotypes can be a laborious task. Auxins such as 2,4-D, 2,4,5-T and Picloram, play a key role in the induction of somatic embryogenesis and it usually necessary to test critically the different types and concentrations of auxin in order to establish protocols which are effective with a range of genotypes from the same species.

3.1 THE EFFECT OF 2,4-D ON SOMATIC EMBRYOGENESIS INDUCTION FROM AXILLARY BUDS OF EIGHT SWEET POTATO GENOTYPES

This experiment was designed to investigate the effects of two concentrations of 2,4-D (5 and 10μM) on the induction of embryogenic tissues from axillary buds of eight genotypes (865M, 1023M, 30MT, 209M, 207M, 132M, TIB10 and Papota) of sweet potato.

For each combination of 2,4-D concentration and genotype, eight axillary buds were placed in each of three replicate petridishes containing MS medium with 0.06M sucrose and the appropriate concentration of 2,4-D. The cultures were maintained at 25°C in the light (16h photoperiod) (see Table 3.1 for details) and explants were monitored every 7d, before the final results were recorded after 28d.

Two morphologically distinct types of callus were distinguishable in the first two week following culture initiation. The first callus type to occur was friable, fast growing and non-embryogenic, although the precise response varied with genotype and concentration of auxin. This fast growing callus which was translucent and white to brownish in colour, never became embryogenic under any of the tested conditions. The second callus type, which appeared after 2-4 weeks, consisted of compact, opaque embryogenic tissue with an irregular and nodular surface. It was variable in colour ranging in different genotypes from pale yellow or yellow (30MT, 209M, 207M and 132M), to purple and red (865M, 1023M and Papota), or to cream and red (TIB10). The surface of the embryogenic callus became convoluted within 10d, eventually giving rise to clusters of globular units of immature embryos (Plate 3A&B). Mature heart-or torpedo- shaped embryos occasionally appeared directly from the surface of the embryogenic tissue in the presence of 2,4-D, particularly in genotype TIB10, but usually such embryos were produced following transfer of the embryogenic tissue to auxin-free medium.

The data presented in Table 3.1 show that embryogenic tissue was produced by all genotypes. However, there were significant differences between the responses of the genotypes ($p<0.01$), among which genotypes TIB10 and 30MT were the most responsive. Also, there were also significant differences ($p<0.01$) between the effects of the two concentration of 2,4-D, with 5μM producing better responses with six of the genotypes.

Table 3.1 Effect of 2,4-D on somatic embryogenesis induction from axillary buds of eight sweet potato genotypes

Genotype	Embryogenic response (%)	
	2,4-D (μM)	
	5	10
865M	12	4
1023M	13	4
30MT	44	37
209M	17	4
207M	8	20
132M	4	16
TIB10	52	54
Papota	25	16

n=24

1. Shoot meristem-tip size : 0.2 – 0.5mm
2. Basal medium: MS + 0.06M sucrose
3. Replication: 3 petri dishes x 8 axillary buds (n=24)
4. Incubation conditions : 25°C/16h photoperiod (70μMm^{-2} s^{-1} PAR)
5. Incubation period : 28d

3.2 THE EFFECT OF 2,4-D CONCENTRATIONS ON THE INDUCTION OF SOMATIC EMBRYOGENESIS FROM AXILLARY BUDS OF EIGHT SWEET POTATO GENOTYPES

Following the demonstrations in experiment 3.1 that all of the tested genotypes produced an embryogenic response in the presence of 2,4-D, the experiment was repeated with a

wider range of 2,4-D concentrations (1,5,10,25 and 50μM) to determine the optimal 2,4-D concentration.

For each combination of 2,4-D concentration and genotype, eight axillary buds were placed in each of three replicate petri dishes containing MS medium with 0.06M sucrose and the appropriate concentration of 2,4-D. The cultures were maintained at 25°C in the light (16h photoperiod) (see Table 3.2 for details) and explants were monitored every 7d, before the final results were recorded after 28d.

The morphological characteristics of callus formation (non-embryogenic and embryogenic) have been described in Section 3.1.

The data presented in Table 3.2 show that embryogenic tissue was produced by all genotypes. However, there were significant differences between the responses of genotypes ($p<0.01$), and genotypes TIB10, 30MT, 865M, 1023M and Papota were the most responsive. Also, there were significant differences ($p<0.001$) between the effects of the five concentrationS of 2,4-D, with 5μM and 10μM producing better responses with all the genotypes.

Table 3.2 Effect of 2,4-D on somatic embryogenesis induction from axillary buds of eight sweet potato genotypes

Genotype	Embryogenic response (%)				
	2,4-D (μM)				
	1	5	10	25	50
865M	4	56	55	3	0
1023M	16	56	19	16	5
30MT	0	52	58	5	0
209M	8	8	17	10	0
207M	16	25	21	10	0
132M	0	4	24	0	0
TIB10	0	44	64	8	0
Papota	0	46	31	4	0

n=24

1 Shoot meristem-tip size : 0.2 – 0.5mm

2 Basal medium: MS + 0.06M sucrose

3 Replication: 3 petridishes x 8 axillary buds (n=24)

4 Incubation conditions : 25°C/16h photoperiod (70μMm^{-2} s^{-1} PAR)

5 Incubation period : 28d

3.3 THE EFFECT OF 2,4,5-T ON THE INDUCTION OF SOMATIC EMBRYOGENESIS FROM AXILLARY BUDS OF EIGHT SWEET POTATO GENOTYPES

In experiments 3.1 and 3.2 it was shown that axillary buds from all of the tested genotypes produced embryogenic tissues in response to 2,4-D in the 5 to 10μM concentration range. The actual proportion, however, of responding buds varied with three of the genotypes showing responses of 25% or less. It was decided therefore, to test the responses of the same range of the genotypes to three different concentrations (1, 5 and 10μM) of an alteration auxin 2,4,5-T.

For each combination of 2,4,5-T concentration and genotype, eight axillary buds were placed in each of three replicate petridishes containing MS medium with 0.06M sucrose and the appropriate concentration of 2,4,5-T. The cultures were maintained at 25°C in the light (16h photoperiod) (see Table 3.3 for details) and explants were monitored every 7d, before the final results were recorded after 28d.

The data presented in Table 3.3 and the accumulated analysis of deviance (appendix 1) show that embryogenic tissue was produced by all genotypes. However, there were significant differences between the responses of genotypes ($p<0.01$), and genotypes 209M, 1023M and Papota were the most responsive (52-67%). Also, there were also significant differences ($p<0.001$) between the effects of the three concentration of 2,4,5-T, with 5μM and 10μM producing better responses with all the genotypes.

In comparison with experiments 3.1 and 3.2, two of the genotypes which gave poor responses to the 2,4-D (209M and 207M) showed improved responses to 2,4,5-T. But genotype 132M showed no improvement and the response of 30MT declined.

Table 3.3 Effect of 2,4,5-T on somatic embryogenesis induction from axillary buds of eight sweet potato genotypes

Genotype	Embryogenic response (%)		
	2,4,5-T (μM)		
	1	5	10
865M	0	29	41
1023M	25	58	40
30MT	0	21	20
209M	12	67	35
207M	8	37	37
132M	8	5	24
TIB10	4	40 28	45 25
Papota	50	52	45

n=24

1 Shoot meristem-tip size : 0.2 – 0.5mm

2 Basal medium: MS + 0.06M sucrose

3 Replication: 3 petridishes x 8 axillary buds (n=24)

4 Incubation conditions : 25°C/16h photoperiod (70μMm^{-2} s^{-1} PAR)

5 Incubation period : 28d

3.4 THE EFFECT OF PICLORAM ON THE INDUCTION OF SOMATIC EMBRYOGENESIS FROM AXILLARY BUDS OF EIGHT SWEET POTATO GENOTYPES

Following the demonstrations in experiments 3.1-3.3 that the tested range of sweet potato genotypes produced variable embryogenic responses to the auxin 2,4-D and 2,4,5-T, a further experiment was designed to determine their response to two concentrations (5 and 10μM) of a third auxin, Picloram.

The appearance of embryogenic tissue differ in size and qua lity (not shiny) than the tissue produced in 2,4-D or 2,4,5-T. For each combination of Picloram concentration and genotype, eight axillary buds were placed in each of three replicate petri dishes containing MS medium with 0.06M sucrose and the appropriate concentration of Picloram. The cultures were maintained at 25°C in the light (16h photoperiod) (see Table 3.4 for details) and explants were monitored every 7d, before the final results were recorded after 28d.

The data presented in Table 3.4 show that embryogenic tissue was produced by seven of the genotypes (12-45%). There were significant differences between the responses of genotypes ($p<0.01$), with genotypes 1023M, 30MT, Papota and 207M being the most responsive (25-45%). Overall, the responses of the genotypes to Picloram were lower than with either 2,4-D or 2,4,5-T. There were no significant differences between the effects of the two concentrations of Picloram.

3.5 THE EFFECT OF AXILLARY BUD LOCATION ON THE INDUCTION OF SOMATIC EMBRYOGENESIS FROM EIGHT SWEET POTATO GENOTYPES

Following the demonstrations in experiments described in sections 3.1-3.4 that all of the tested genotypes produced embryogenic response in the presence of the auxin 2,4-D,

Table 3.4 Effect of Picloram on somatic embryogenesis induction from axillary buds of eight sweet potato genotypes

Genotype	Embryogenic response (%)	
	Picloram (μM)	
	5	10
865M	6	4
1023M	45	30
30MT	30	13
209M	0	19
207M	12	25
132M	0	0
TIB10	10	12
Papota	30	4

n=24

1 Shoot meristem-tip size : 0.2 – 0.5mm

2 Basal medium: MS + 0.06M sucrose

3 Replication: 3 petri dishes x 8 axillary buds (n=24)

4 Incubation conditions : 25°C/16h photoperiod (70μMm^{-2} s^{-1} PAR)

5 Incubation period : 28d

2,4,5-T and Picloram, this experiment was designed to determine the optimal location of buds taken from eight weeks old shoot cultures for induction of somatic embryogenesis. In this and future experiments, it was decided to use 2,4-D as the standard auxin for induction of embryogenic tissue because all of the sweet potato genotypes, with the exception of 209M, under investigation showed positive responses, and neither 2,4,5-T nor Picloram produced significantly improved overall responses.

For each combination of bud location and genotype, eight axillary buds from four different locations were placed in each of three replicate petridishes containing MS medium with 0.06M sucrose and 5μM 2,4-D and maintained at 25°C in the light (16h photoperiod) (see Table 3.5 for details). Explants were monitored every 7d and the final results were recorded after 28d.

The data presented in Table 3.5 indicate that embryogenic tissue was produced by all genotypes. There were significant differences between the responses of genotypes ($p<0.001$), and genotypes Papota, TIB10 and 30MT were the most responsive. Also, there were significant differences ($p<0.01$) between the effects of the four axillary bud locations, with the 11-15 nodal portion producing better responses with seven of the genotypes. Since, however, the differences between the responses of the buds from different locations in the various genotypes were not always large, it was decided for practical reasons that in future experiments buds taken from all locations would be utilized after pooling randomization.

3.6 THE EFFECT OF SUCROSE ON THE PROLIFERATION OF EMBRYOGENIC AGGREGATES FROM TWO SWEET POTATO GENOTYPES

Since in other species, sucrose has been shown to have an influence on the embryogenic response, this experiment was designed to investigate the effects of sucrose on the

Table 3.5 The effect of axillary bud location on the induction of somatic embryogenesis from axillary buds of eight sweet potato genotypes

Genotype	Embryogenic response (%)			
	Nodal position (from apex)			
	1-5	6-10	11-15	16-20
865M	8	8	33	21
1023M	4	5	17	14
30MT	42	29	31	25
209M	4	9	13	10
207M	3	3	4	0
132M	4	4	6	0
TIB10	25	42	44	29
Papota	25	50	54	42

n=24

1 Nodal positions (from apex):

1-5 nos

6-10 nos

11-15 nos

16-20 nos

2 Age of plant : 8 weeks

3 Size of nodal position

4 Basal medium: MS + 0.06M sucrose + 5μM 2,4-D

5 Replication: 3 petri dishes x 8 axillary buds (n=24)

6 Incubation conditions : 25°C/16h photoperiod (70μMm^{-2} s^{-1} PAR)

7 Incubation period : 28d

proliferation of embryogenic tissues in two sweet potato genotypes. The embryogenic aggregates used for this experiment were taken from cultures which had been grown for 28d on MS medium and 5μM 2,4-D.

For each combination of sucrose concentration (0.06M and 0.1M) and genotype (865M and 30MT), ten embryogenic aggregates (1.0-2.0mm in diameter) were placed in each of four replicate petridishes containing MS medium with 5μM 2,4-D and the appropriate concentration of sucrose, and maintained at 25°C in the light (16h photoperiod) (see Fig. 3 A and B for details). The size of embryogenic aggregates was determined on a fresh weight basis over a period of 28d.

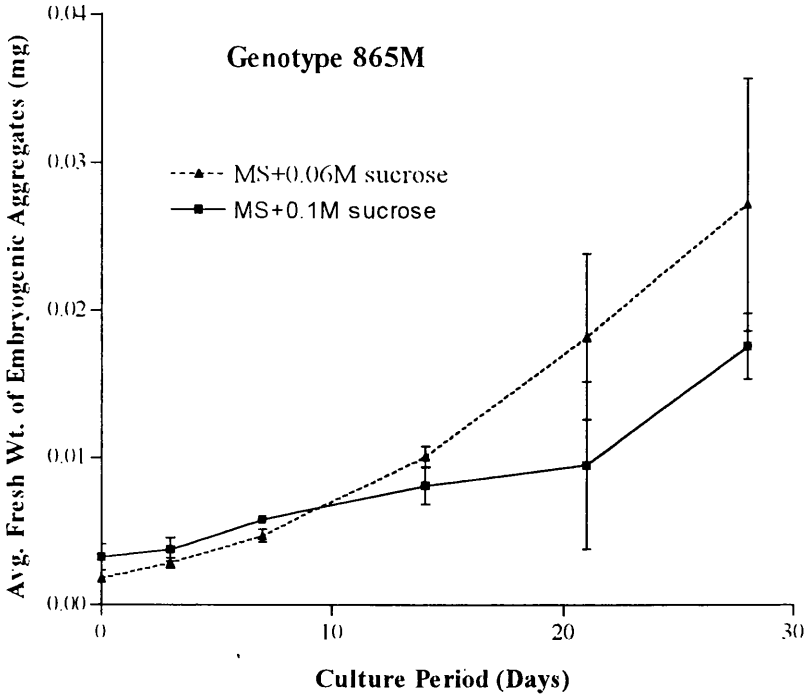
The data presented in Fig 3 show that embryogenic tissue was produced by both genotypes, and there were significant differences between responses of genotypes, with genotype 865M being the more responsive. Also, there were significant differences between the effects of the two concentration of sucrose, with 0.1M producing the better responses with both of the genotypes after initiation for more than 7d.

3.7 THE EFFECT OF DIFFERENT EXPOSURES TO 2,4-D ON PLANTLET FORMATION FROM EMBRYOGENIC AGGREGATES WITH FOUR SWEET POTATO GENOTYPES

Since there can be considerable differences between the effects of an auxin on the initiation of embryogenic tissue and on the subsequent development of somatic embryos, this experiment was designed to investigate the effects of different exposures to 2,4-D on plantlet development from embryogenic aggregates of four sweet potato genotypes. The embryogenic aggregates used for this experiment were taken from cultures which had been grown after 15d subculrure on MS medium + 5μM 2,4-D.

For each combination of 2,4-D treatment (T_1-T_5) and genotype (865M, 1023M, 30MT and TIB10), ten embryogenic aggregates (1.0-2.0mm in diameter) were placed in each of three replicate petridishes containing MS medium with 0.06M sucrose and 1μM 2,4-D and maintained at 25°C in the light (16h photoperiod) for periods between 7 and 14d (see Table 3.7 for details). After hormonal media treatment, embryogenic aggregates were transferred to petridishes containing MS medium with 0.06M sucrose and incubated for a further 28d under the same conditions. The plantlet development data was monitored every 7d and the final results were recorded after 28d.

The data presented in Table 3.7 indicate that plantlets were produced by all genotypes. There were significant differences between the responses of genotypes ($p<0.05$), with genotype 1023M being the most responsive. Also, there were significant differences ($p<0.01$) between the effects of the 2,4-D treatments, with T_3 treatment (21d exposure to 2,4-D) producing the best response (40-60%) with three of the genotypes (1023M, 30MT and TIB10).



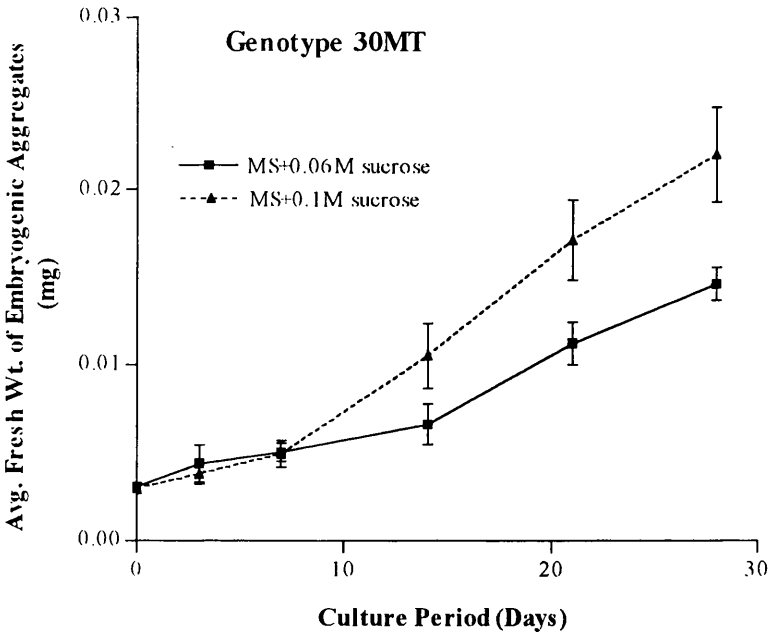


Fig. 3. Effect of sucrose on the proliferation of embryogenic aggregates from two sweet potato genotypes. Error bars represent standard deviation on the means of 4 replicates.

Table 3.7 Effect of different exposures to 2,4-D on plantlet formation from embryogenic aggregates with four sweet potato genotypes

Media treatment(d)	Plantlet development (%)											
	865M			1023M			30MT			TIB10		
	S	R	S+R	S	R	S+R	S	R	S+R	S	R	S+R
0	40	25	10	80	40	26	10	7	0	40	30	5
7	50	38	17	90	60	~~60~~ 40	13	5	3	70	50	13
14	55	40	22	92	73	52	25	7	4	80	60	23
21	60	33	13	80	75	60	40	18	9	90	73	28
28	32	20	10	40	30	15	55	10	7	10	5	3

n=30

1. Media treatments:

i) MS + 0.06M sucrose (28d)

ii) MS+ 0.06 sucrose + 1μM 2,4-D (7d)→MS + 0.06M (28d)

iii) MS+ 0.06 sucrose + 1μM 2,4-D (14d)→MS + 0.06M (28d)

iv) MS+ 0.06 sucrose + 1μM 2,4-D (21d)→MS + 0.06M (28d)

v) MS+ 0.06 sucrose + 1μM 2,4-D (28d)→MS + 0.06M (28d)

1 Size of embryogenic aggregate : 2.0- 4.0mm

2 Basal medium: MS

3 Replication: 3 petri dishes x 8 axillary buds (n=30)

4 Incubation conditions : 25°C/16h photoperiod (70μMm^{-2} s^{-1} PAR)

5 Incubation period : 28d

3.8 THE EFFECT OF SUCROSE CONCENTRATION AND EMBRYOGENIC AGGREGATE SIZE ON PLANTLET FORMATION FROM EMBRYOGENIC AGGREGATES OF TWO SWEET POTATO GENOTYPES

Because of the number of treatments (two sucrose treatments x three embryogenic aggregate sizes) investigated with each of two genotypes, it was necessary to deal with the individual genotypes in separate experiments. The primary aim, however, was to investigate the effects of sucrose and embryogenic aggregate size on subsequent plantlet formation with two sweet potato genotypes. 5μM 2,4-D.

For each combination of sucrose treatment and size of embryogenic aggregate, ten embryogenic aggregates were placed in each of three replicate petridishes containing MS medium with 0.06M and maintained at 25°C in the light (16h photoperiod) for each treatment (see Table 3.8 for details). Plantlet development was monitored every 7d and the final results were recorded after 28d.

Table 3.8 Effect of sucrose concentration and embryogenic aggregate size on plantlet formation from embryogenic aggregates of two sweet potato genotypes

Sucrose treatment	Aggregate diameter (mm)	Plantlet development (%)					
		865M			209M		
		Shoot only	Root only	Shoot and root	Shoot only	Root only	Shoot and root
T_1	1.0-2.0	90	40	40	83	20	20
	2.0-3.0	80	30	30	87	15	15
	3.0-4.0	65	20	20	60	10	10
T_2	1.0-2.0	92	50	50	90	50	50
	2.0-3.0	85	40	40	87	30	30
	3.0-4.0	70	25	25	57	10	10

n=30

1. Sucrose treatments:

T_1= 0.06M

T_2= 0.09M

1 Basal medium: MS

2 Replication: 3 petri dishes x 10 embryogenic aggregates (n=30)

3 Incubation conditions : 25°C/16h photoperiod (70μMm^{-2} s^{-1} PAR)

4 Incubation period : 28d

3.8.1 GENOTYPE 865M

The data presented in Table 3.8 and the accumulated analysis of deviance (. not shown .) indicate that plantlets were produced by the embryogenic aggregates from all of the size categories. There were significant differences between the responses of aggregates from the different categories ($p<0.05$), and the 1.0-2.0mm were the most responsive. Also, there were significant differences ($p<0.05$) between the effects of sucrose treatments, with T_2 (0.09M) treatment producing better response with each aggregate size.

3.8.2 GENOTYPE 209M

In general terms, the responses with genotype 209M were the same as those for genotype 865M, except that overall plantlet production was lower.

3.9 THE EFFECT OF DIFFERENT PLANT GROWTH REGULATORS ON PLANTLET PRODUCTION FROM EMBRYOGENIC AGGREGATES OF TWO SWEET POTATO GENOTYPES

The results in experiment 3.7 and 3.8 showed that all of the tested genotypes produced plantlets from embryogenic aggregates in the presence of 2,4-D with responses ranging according to the time of exposure to the 2,4-D and also according to the aggregate size. This experiment, therefore, was conducted to investigate the effects of a wider range of plant growth regulators (GA_3, NAA, BAP, IBA and GA_3 + NAA), applying the procedure used in experiment 3.7 to two genotypes (865M and 209M). Because of the number of treatments (five plant growth regulator treatments x four culture periods), it was necessary to deal with the individual genotypes in separate experiments. The embryogenic aggregates used for this experiment were taken from cultures which had been grown for 28d on MS medium + 5μM 2,4-D.

For each combination of plant growth regulator and treatment and culture period, ten embryogenic aggregates (1.0-2.0mm in diameter) were placed in each of three replicate petridishes containing MS medium with 0.06M and the appropriate plant growth regulator and maintained at 25°C in the light (16h photoperiod) for each culture period (see Table 3.9 for details). After these treatments, the embryogenic aggregates were transferrred to petridishes containing MS medium with 0.06M sucrose and incubated for a further 28d. The maturation and germination date were monitored every 7d and the final results were recorded after 28d.

3.9.1 GENOTYPE 865M

The data presented in Table 3.9 show that the shorter periods of exposure (7d and 14d) to the growth regulator, in each case, led to a higher production of mature embryos than in the control. There were significant diffeences between the effects of the different exposure periods to the growth regulators ($p<0.01$), with the 7d period leading to the most productive response. Also, there were significant differences ($p< 0.01$) between the effects of the different growth regulators on subsequent platlet formation, with the 7d or 14d periods of exposure to GA_3 producing the best responses. In comparison with the control, these shorter periods of exposure (7d and 14d) to any of the growth regulators led to a higher proportion of embryogenic aggregates yielding one or more shoots.

3.9.2 GENOTYPE 209M

The data presented in Table 3.10 shows that the shorter periods of exposure (7d and 14d) to the growth regulators led, in each case, to a higher production of mature embryos in comparison with the control. The analysis of deviance (not shown) show that there were significant differences between the effects of the different exposure periods to the

growth regulators ($p<0.01$) with the 7d culture period leading to the most productive response. Also, there were significant differences ($p<0.01$) between the effects of the different growth regulators on subsequent plantlet formation, with the 7d exposure to GA_3 producing the best response. Three of the growth regulator treatment (NAA, BAP and GA_3 + NAA) produced responses which were no better or worse than the control.

Table 3.9 Effect of different plant growth regulators on plantlet production from embryogenic aggregates of sweet potato genotype 865M

Plant growth regulator	Culture period in PGRs (d)	Aggregates producing mature embryos(%)	Plantlet development from embryogenic aggregates (%)		
			Shoot only	Root only	Shoot and root
MS_2 (control)	28	60	55	40	25
GA_3 (1μM)	7	95	70	55	50
	14	80	60	35	40
	21	75	45	20	15
	28	65	40	10	5
NAA(1μM)	7	80	30	40	20
	14	90	45	55	35
	21	70	15	20	25
	28	60	5	10	5
BAP(1μM)	7	85	25	30	20
	14	70	35	40	30
	21	65	17	10	7
	28	60	10	5	0
IBA(1μM)	7	95	56	60	40
	14	80	45	40	35
	21	65	30	25	15
	28	60	15	10	5
GA_3 + NAA (1μM)	7	80	20	35	20
	14	90	45	26	38
	21	70	30	20	10
	28	65	10	5	0

n=30

1. Culture treatments:

MS + 0.06M sucrose appropriate plant growth regulator (1μM) for indicated time (7-28d), before transfer to MS + 0.06M sucrose for 28d

2 Basal medium: MS + 0.06M sucrose

3 Replication: 3 petri dishes x 10 embryogenic aggregates (n=30)

4 Size of embryogenic aggregate: 1.0-2.0mm

5 Incubation conditions : 25°C/16h photoperiod (70μMm^{-2} s^{-1} PAR)

6 Incubation period : 28d

Table 3.10 Effect of different plant growth regulators on plantlet production from embryogenic aggregates of sweet potato genotype 209M

Plant growth regulator	Culture period in PGRs (d)	Aggregates producing mature embryos(%)	Plantlet development from embryogenic aggregates (%)		
			Shoot only	Root only	Shoot and root
MS_2 (control)	28	50	45	30	20
GA_3 (1μM)	7	80	53	47	50
	14	75	40	30	23
	21	60	10	23	10
	28	50	10	13	5
NAA(1μM)	7	75	23	43	20
	14	85	7	20	10
	21	70	7	17	3
	28	70	10	16	5
BAP(1μM)	7	75	10	25	20
	14	60	7	20	16
	21	50	10	17	13
	28	50	13	20	10
IBA(1μM)	7	85	23	43	37
	14	70	10	13	27
	21	90	3	17	7
	28	50	5	13	0
GA_3 + NAA (1μM)	7	75	10	33	10
	14	85	20	40	20
	21	60	15	37	0
	28	40	10	27	0

n=30

For experimental details see Table 3.9

3.10: DISCUSSION

The first objective of this project was to produce embryogenic tissues from a wider range of sweet potato genotypes (see Chapter 2, Table 2.1 for details) and at a higher frequency than had been reported by other workers (see chapter 1, Section 1.2.2.2). This is important if the embryogenic systems are to be used in conjunction with either genetic transformation or mass propagation programs. Also, as far as the immediate project was concerned, it was important that the embryogenic tissues which were to be used in the subsequent cryopreservation studies should have been derived from a range of genotypes. The second, important aim was to apply successful cryopreservation techniques for embryogenic tissue to shoot meristems for genetic conservation purposes where, again, applicability to a wide range of germplasm is of prime importance.

To investigate the effect of 2, 4-D, eight genotypes were tested for the induction of embryogenic tissues. All the genotypes produced positive responses with one or the other treatment. Four of the genotypes produced relatively high frequency responses, ranging from 56-64% of the explants under optimal conditions. The remaining genotypes were less responsive, ranging from 17-44% (see Section 3.2 for details).

It was also shown that growth regulator concentration has strong influence on the induction of embryogenic tissues. Among the five concentrations of 2, 4-D tested, 5μM was in general the most effective in producing good quality (shiny and globular tissues) and quantities of embryogenic tissues from all genotypes (see Section 3.1 and 3.2 for details).

For comparison, the use of 2, 4, 5-T for the induction of embryogenic tissues in a similar range of genotypes was investigated in Section 3.3. Embryogenic tissues were again

produced with all of the genotypes, with responses ranging from 24-67%. As with 2, 4-D, the embryogenic response of genotypes to 2, 4, 5-T was therefore quite variable and, again 5μM represented the optimal concentration of the growth regulator. significantly, however, the different genotypes responded differently to the compounds with, for example genotype 209M producing the highest induction rate (67%) with 2, 4, 5-T and a very low rate 8% with 2, 4, D (see Sections 3.2 and 3.3 for details). It is evident, therefore, if high induction rates are important for any particular application, it maybe necessary to select an appropriate growth regulator for that particular purpose.

In order possibly to widen further the choice of growth regulator for the induction of embryogenic tissue, a further experiment was carried with the eight sweet potato genotypes and a third growth regulator, Picloram (see Section 3.4 for details). The four most responsive genotypes (1023M, Papota, 30MT and 207M) showed induction rates in the range 25-45%, which were lower than those obtained with either 2, 4-D or 2, 4, 5-T. The remaining genotypes produced much lower induction rates, in the range 6-19%, and genotype 132M showed no response. The optimal concentration of Picloram was, again, 5μM, but it is unlikely that Picloram would be used in preference to 2, 4-D or 2, 4 5-T for induction embryogenic tissues in sweet potato.

Induction of embryogenic tissues in sweet potato was first demonstrated by Tsey and Tseng (1979) who reported the effects of the growth regulators IAA, 2, 4-D, kinetin and different basal media on embryogenic callus formation from anthers of five genotypes. Embryogenic callus was induced from anthers of all of the genotypes and a high response was obtained from the genotype Tainung Hsin No. 31 when cultured on MS medium

containing 2mg l^{-1} 2, 4-D and 2 mg l^{-1} kinetin. The induction rate for the embryogenic tissue was not, however, reported.

Jarret et al (1984) investigated the effect of a range of 2, 4-D concentrations (0.1, 0.3 1.0 and 3.0 mg l^{-1}) on the induction rate of embryogenic tissues excised axillary buds of nine sweet potato genotypes. six of the genotypes produced embryogenic tissues at frequencies between 10 and 30% and the induction rate was strongly affected by both the concentration of 2, 4-D and genotype, as well as the original position of the axillary bud on the shoot.

Liu and Cantliffe (1984) reported the embryogenic responses of two genotypes, GaTG3 and White Star, using different sources of explants. They found that leaf primordia explants produced embryogenic callus at much higher rates than shoot tips, stems and roots. Although, the leaf explants were the most responsive among the explant that were tested, axillary buds were used as explants in all of the later studies by these workers.

In a second study, the investigations concerning the induction of embryogenic tissue were carried out with the genotype white star. Embryogenic tissue was induced by culturing meristem tips and two leaf primordia on MS medium supplemented with 10μM 2, 4-D, but the frequency of induction of embryogenic tissue was only 19% (Chee and Cantliffe; 1988a).

More recently, an investigation with ten West Indian and Chinese sweet potato genotypes has been reported by Cavalcante-Alves et al (1994). Embryogenic tissues were induced from lateral buds, cultured on MS medium supplemented with 10μM 2,4-D and 0.09M sucrose

overall, however induction frequencies were low with two genotypes showing values of 17% and 15% and the remainder being lower than 10%.

Also, Al-Mazrooei (1996) investigated the embryogeinc responses of 10 genotypes of sweet potato including some used in the present investigation. Axillary buds excised from 10 genotypes were cultured on MS medium supplemented with six 2,4-D concentrations (1, 5, 10, 30, 50 and 100μM) and 0.09M sucrose. Three of the genotypes (Nem, TIBIO and Papota) showed relatively high induction frequencies embryogenic tissues ranged from (46-64%) and one genotype responded at 20% but the remaining six genotypes were completely unresponsive. Five or 10μM 2. 4-D gave the highest induction frequencies.

Al-Mazrooei (1996) also first reported the use of 2, 4-1 2,4,5-T for the induction of embryogenic tissue with the same range of sweet potato genotypes. Eight of the genotypes showed induction frequencies in the range 8-80%, but only three genotypes produced frequencies than 50%, with a further two-genotypes 30-32% and two of the genotypes were completely unresponsive. The 5μM concentration of 2, 4, 5-T produced the best response in terms of the high frequency of embryogenic tissue induction.

Using the growth regulator Picloram, Al-Mazrooei (1996) reported the effect of three concentrations (1, 5 and 10μM) on the induction of embryogeinc tissues with three previously recalcitrant genotypes (Brondal, Jersey Orange and Rose Continental) of sweet potato. Two of these genotypes produced embryogenic tissues at low frequencies (up to 4%), with the 5 or 10μM concentrations.

In comparison with these previous work with sweet potato, the embryogenic induction frequencies obtained in the present investigation with axillary buds cultured on MS

medium supplemented with 5μM 2, 4-D or 2, 4, 5-T represent the highest values for the eight of the genotypes. Also, the relatively high induction frequencies obtained for four of the genotypes with Picloram, represent the highest values that have been obtained with this particular growth regulator.

Since it is possible that the induction rates for embryogenic tissues could be further improved by careful choice of bud explants of a particular developmental age, an investigation was carried out with 2, 4-D and bud explants excise from among the youngest 20 nodes of eight week old shoot cultures (see Section 3.5 for details). Overall, the most responsive buds with eight sweet potato genotypes were those taken from between the 11th and 15th nodes.

The variation in the induction rates with different bud locations was presumably due to differences in their developmental ages, since embryogenic competence has been found to be correlated with the age of explant in other species, such as cereals (Vasil, 1995). Also, the sizes of the explants from the different nodal position is likely to have an effect since the smaller buds are easily damaged during excisions.

In previous work with sweet potato, Jarret et al, (1984) reported in two sweet potato genotypes, differences in the embryogenic responses of the axillary buds according to their position on the donor plant, and they found that out of the youngest nine buds, only the terminal bud and first five axillary buds produced embryogenic tissues with frequencies ranging from 25% to 60%. Al-Mazrooei (1996) however, reported that no significant differences were found in the induction rate of embryogenic tissues with the first eight buds on shoot either genotypes TIBIO or Nem.

Although the present work showed relatively high induction rates for embryogenic tissue with buds taken from the 11-15th nodal position, it was considered that the values obtained were not sufficiently high to justify the very considerable extra work that would have been involved in the routine selection of buds for future experiment. subsequently, therefore, buds were taken randomly from all positions on the shoots, up to the 20th node, and pooled before use. The results did, however, indicate a possible approach to work with recalcitrant sweet potato genotypes.

Although very promising results had been obtained in terms of the induction rates for embryogenic tissues with a relatively diverse range of sweet potato genotypes, it is likely that further experiments could have been made by investigations with other factors such as further growth regulators, different basal medium an possibly different explant sources. It was decide, however, that time did not permit such investigations, and attention was turned to ways of improving the rates of proliferation of embryogenic tissue, once they had been induce, and rates of recovery of plantlets from somatic embryos produced from such tissues. Both of these factors have an important bearing on the usefulness of those tissues for cryopreservation studies for other purposes such as genetic transformation or mass propagation.

Embryogenic cultures are frequently maintained on a medium similar in composition to the induction medium which normally contains an auxin. The successful maintenance methods, however, must at the same time as encouraging the growth of the embryogenic tissue, discourage the growth of more disorganized non-embryogenic tissue, and several factors seem to play an important role in the maintenance of this balance, including growth regulator and subculture regimes. A comprehensive study of this problem could

involve extensive investigations and it was decided, simply, to investigate the influence of sucrose which has been shown by workers with other species to have an effect on the proliferation of embryogenic tissues (see Section 1.2.4 for details). The effect of two concentrations of sucrose (0.06M and 0.1M) were therefore studied with two of the sweet potato genotypes.

The results demonstrated that with both genotypes, more embryogenic tissue was produced over a period of four weeks in the presence of the higher sucrose concentration and the embryogenic tissues were shiny and globular in appearance. Experience has shown that this type of appearance is highly characteristic of healthy embryogenic tissues which are capable of sustained growth.

Verma and Dougall (1977) suggested that in general sucrose appears to be the most effective carbon source for the support of somatic embryogenesis, although many other mono- and disachharides can be successfully employed. Higher sucrose concentration (0.09M) in the induction medium have also been shown to benefit the proliferation and maintenance of embryogenic tissues in gladiolus (Stefnaik, 1994) and sweet potato (Chee and Cantliffe, 1988; Al-Mazrooei, 1996). Sudarmonowati (1990), however suggested that a somewhat lower concentration of sucrose (0.06M) could be used to maintain embryogenic tissues of cassava, since higher concentrations of sucrose (0.12M or 0.18M) caused the majority of clumps of somatic embryos to turn white and opaque, indicating a loss of embryogenic competence which could not be restored by subsequent transfer to regeneration medium.

On the basis of these results, the standard procedure adopted for all of the subsequent experiments involved the use of stock embryogenic cultures, which had been subcultured

every four weeks on to fresh semi-solid MS medium supplemented with 5μM 2,4-D and 0.1M sucrose. In the cryopreservation studies, the shiny, globular appearance that is typical of the growth of healthy embryogenic tissue on this particular medium, was taken as an important early sign of survival following the freezing/thawing processes.

Ultimately, efficient methods for the maturation of the somatic embryos and for subsequent plantlet development are essential if the embryogenic system is to be fully exploited for any practical purposes. These two processes are frequently considered as separate stages in the *in vitro* embryogenic system, possibly with differing medium requirements. Generally speaking, mature embryos are only produced when the growth regulator concentration is considerably reduced below the concentrations employed in the induction and maintenance media, and therefore growth regulators are usually omitted from the maturation medium. The nutrient and growth regulator requirements for plantlet development from the mature embryos, on the other hand, can vary considerably between species (see Section 1.2.4 for details), particularly in relation to balanced root and shoot development.

With the aim, therefore, of optimising plantlet recovery from the embryogenic aggregates, the effects of exposure of the embryogenic tissues to 2,4-D for different periods of time in the maintenance medium, prior to transfer to auxin-free maturation medium were investigated (see Section 3.7), together with the effects of embryogenic aggregate size (see Section 3.8). It was decided to investigate these particular factors because the duration of exposure to a growth regulator in the maturation medium can influence the amount of growth regulator carried with tissue into the maturation medium; also, competitive interactions between developing embryos in embryogenic aggregates

can influence the process of maturation. These experiments were followed by an investigation of the effects of a range of different growth regulators on the maturation of embryogenic aggregates and subsequent plant recovery from the mature somatic embryos (see Section 3.9).

The results of the first study demonstrated that the length of the period of exposure to the medium containing 1μM 2,4-D prior to transfer to the growth regulator free medium for embryo development did not affect the number of embryos that were produced but it did affect the number of embryos that regenerated into plantlets. Embryogenic aggregates exposed to 2,4-D for 21d showed higher percentages of conversion to plantlets than those which were subjected to the same medium for shorter periods of time. This may be because the longer exposure of embryogenic tissue to the medium containing 2,4-D resulted in the gradual depletion of the 2,4-D from the tissue and the medium, with the result that less of the growth regulator was carried over with the tissue to the regeneration medium where it would have an inhibitory effect on plantlet development (Plate 4).

In the second study concerning the effects of embryogenic aggregate size (1.0-2.0, 2.0-3.0, 3.0-4.0 mm in diameter) combined with different sucrose concentration (0.06M and 0.09M) on the auxin-free regeneration medium, there was no evidence of an effect on the numbers of embryos that were produced; however, the smaller embryogenic aggregate size combined with the higher sucrose concentration produced the highest percentages of conversion to plantlets.

The results of the third study demonstrated that growth regulators applied alone or in combination (GA_3, NAA, BAP, IBA and GA_3 + NAA) before transfer to growth regulator-free regeneration medium did not affect the maturation of somatic embryo, but

they did affect the subsequent development of embryos into plantlets. The highest percentages of conversion to plantlets occurred in the presence of GA_3 and the period of exposure to the various growth regulators also affected the proportion of somatic embryos that subsequently regenerated into plantlets; embryogenic aggregates exposed for 7d or 14d showed higher percentages of conversion to plantlets than was obtained with longer exposures (Plate 4).

The amount of published work concerning the effect of growth regulators on the maturation of sweet potato embryos and plantlet regeneration is rather limited. Chee and Cantliffe (1988) reported that the production of somatic embryos and regeneration to plantlets was achieved by transferring tissue to a medium devoid of growth regulators but apparently no detailed study of this process was made. Chee et al (1990) studied the effect of growth regulators BAP and NAA with sucrose on plant regeneration from somatic embryos. The best plant development was achieved by the use of 0.1μM NAA in combination with MS medium supplemented with 0.09M sucrose. Reducing the sucrose concentration from 0.09M to 0.05M in basal medium increased the frequency of plantlet development to 32% of the treated embryos. Later, Cavalcante-Alves et al (1994) described a more complicated procedure in which compact embryogenic calli were first transferred to a medium containing 10μM 2,4-D or 1μM BAP for the production of globular embryos, and then to a second maturation medium containing 0.01μM 2,4-D and 0.01μM kinetin for the efficient production of cotyledonous embryos, prior to transfer to growth regulator-free medium for plantlet formation. More recently, Al-Mazrooei (1996) investigated the effects of different combinations of GA_3, BAP and NAA on embryo formation and plantlet development from sweet potato embryogenic tissue of the Nem genotype and concluded that the highest plantlet development of embryos was achieved

with an exposure of 5d to matureation medium containing 10μM GA_3; 0.5μM BAP and 0.5μM NAA prior to transfer to growth regulator-free medium.

In the present study, which was closest in design to that of Al-Mazrooei (Loc; cit.), it was shown that there was little or no advantage in using any of a range of growth regulators, including GA_3, NAA and BAP and IBA in the maturation medium instead of 2,4-D prior to transfer to the growth regulator-free plant recovery medium, except that the plantlet produced in the presence of GA_3 were greener and more vigorous. This may have been because different genotypes were investigated.

No previous work has been reported concerning the effect of aggregate size and sucrose on plantlet development from somatic embryos in sweet potato. In this study, a smaller aggregate size in the range tested (1-2 to 3-4 mm diameter) was found to improve the conversion rate of the embryo into plantlets when they were cultured on growth regulator-free medium with a higher sucrose level. The reason for the enhanced plantlet development in the smaller aggregates are not clear but it is possible that there is reduced competition for the nutrients and/or there is less accumulation of inhibitory substances.

At this stage of investigation, it was concluded that a relatively efficient system for the induction of somatic embryogenesis and subsequent plantlet recovery had been developed for the group of eight sweet potato genotypes that was to be used in the cryopreservation studies. It was recognized that further improvements could possibly have been made, but the system described above was considered to be adequate, at least to initiate the cryopreservation investigation. If required, additional improvements might be based on the use of activated charcoal to reduce further the concentration of growth regulators in the embryo maturation medium, as used with many culture, including date

palm (Tisserat and De Mason, 1980) and *Carcia stipulata* (Litz and Conover, 1980). Also, a reduction in the concentration of the basal medium particularly during the plantlet development stages might be beneficial. Greater efficiency in the embryo maturation stage might, on the other hand, be obtained by the further reduction in aggregate size that would be achieved if the embryogenic tissue could be dispersed in shaken liquid medium.

Plates 3A and 3B: Induction of embryogenic tissues in eight sweet potato genotypes

Shoot meristems (0.2-1.0mm in diameter) were cultured on MS medium supplemented with 5μM 2,4-D and 0.09M sucrose and subcultured after 3-4 weeks

3.A: Genotypes (all x20)

a. 865M

b. 1023M

c. TIB10

3.B: Genotypes (a - d x20, e x30)

a. 30MT

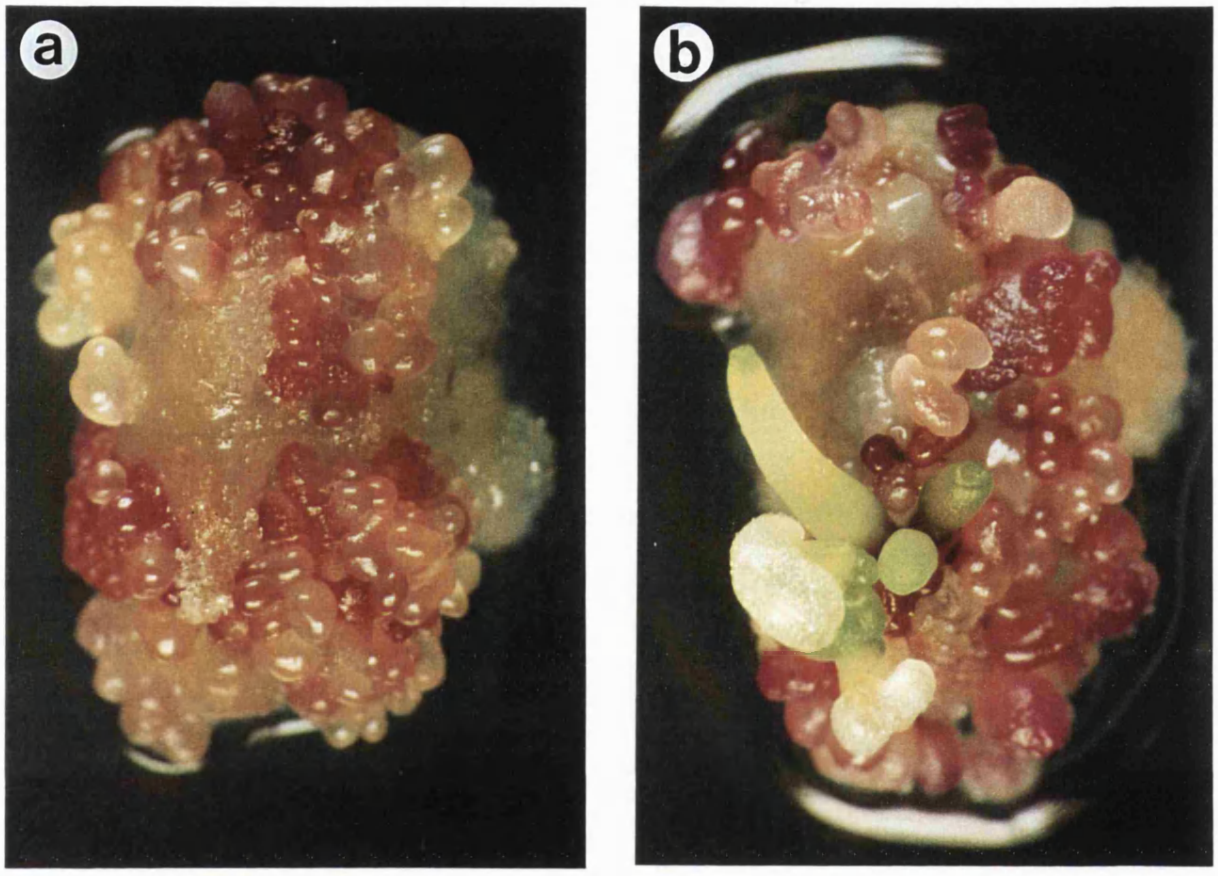
b. 207M

c. 209M

d. 132M

e. Papota

3.A



3.B

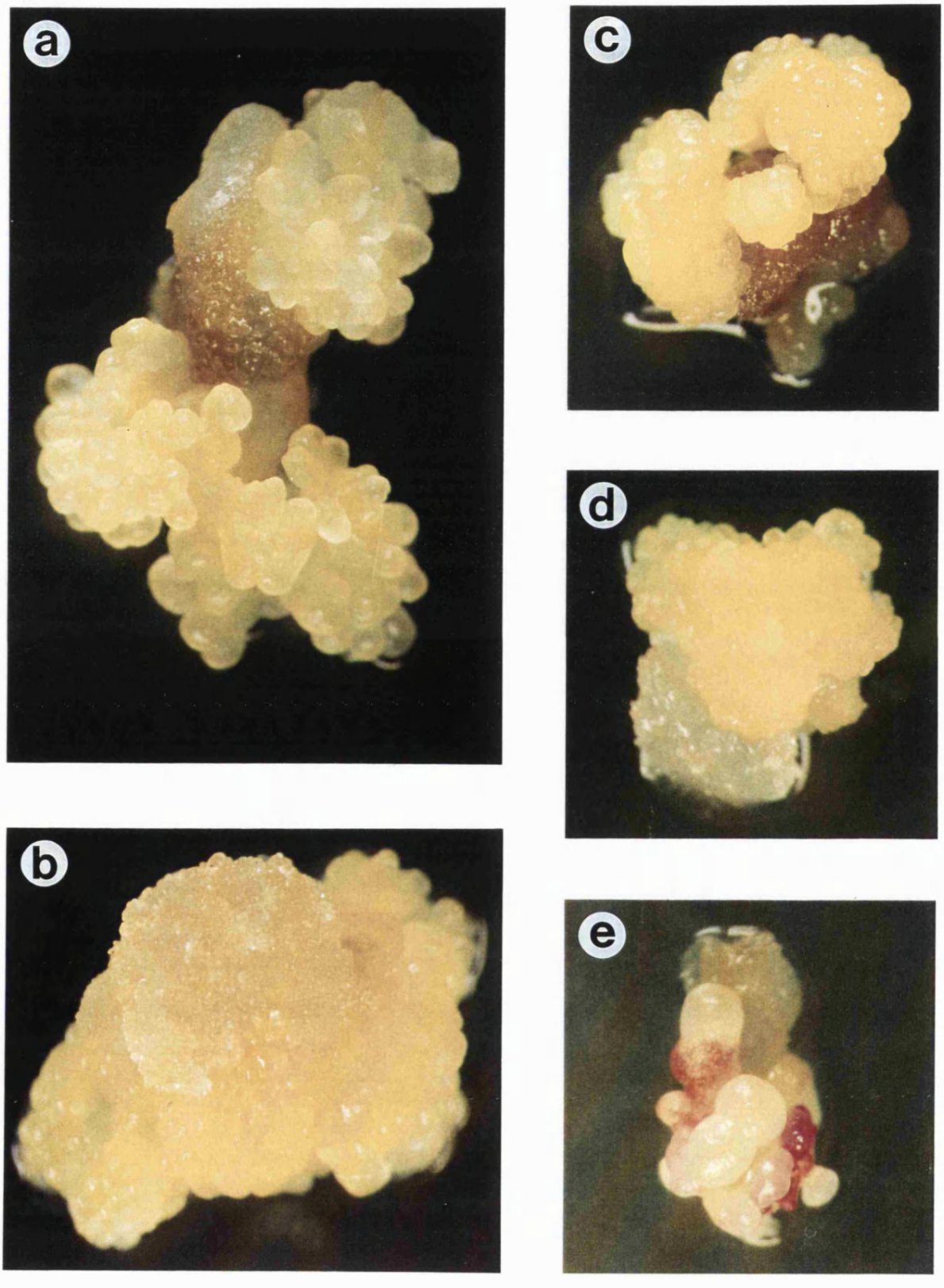
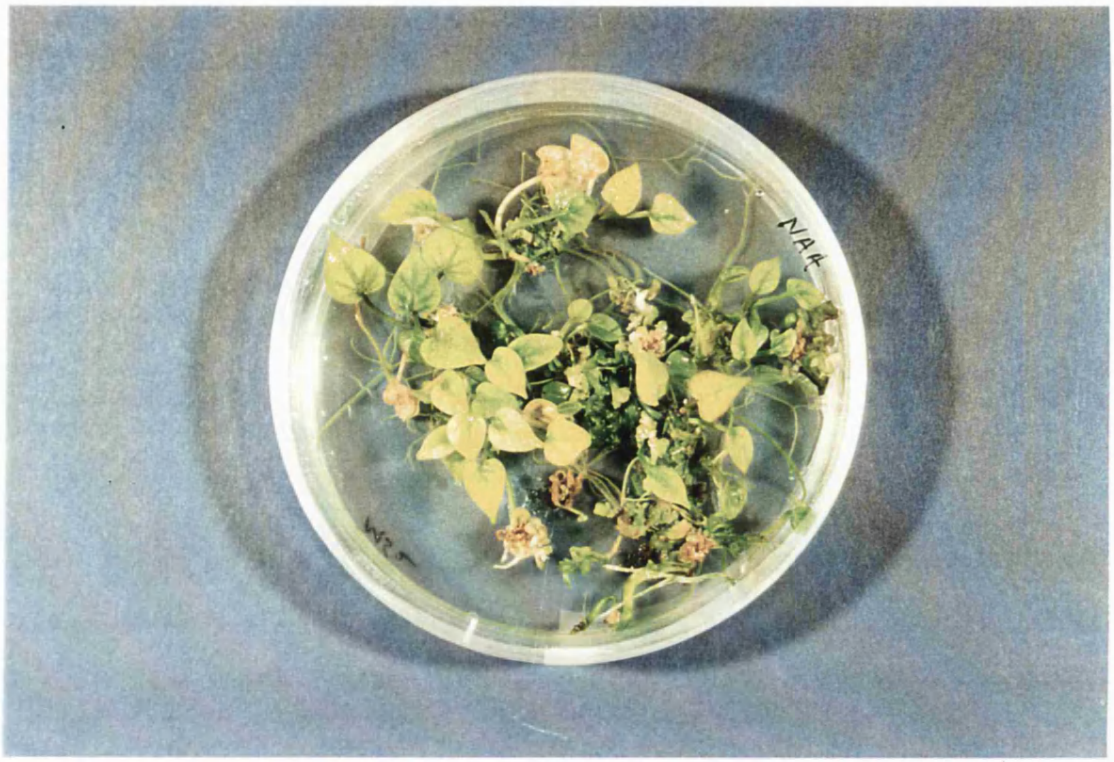


Plate 4: Plant regeneration from embryogenic aggregates

4.A: Embryogenic aggregates (1.0-2.0mm in diameter) of genotypes 865M after growth on maturation medium (MS medium supplemented with 1μM GA_3 and 0.06M sucrose) for one week before transfer to regeneration medium (auxin-free MS medium supplemented with 0.09M sucrose) for four weeks.

4.B: Embryogenic aggregates of genotype 1023M after growth on maturation medium (MS medium supplemented with 1μM 2,4-D and 0.06M sucrose) for two weeks before transfer to regeneration medium (auxin-free MS medium supplemeted with 0.09M sucrose) for four weeks.

4.A



4.B

4. CRYOPRESERVATION OF SWEET POTATO EMBRYOGENIC TISSUES

4.0 CRYOPRESERVATION OF SWEET POTATO EMBRYOGENIC TISSUES

To be of practical value, cryopreservation techniques must be relatively simple and applicable to a wide range of genotypes. The production and maintenance of good quality embryogenic tissues suitable for use in transformation protocols, and the transgenic lines derived from such tissues require both time and skill. Such repetitive work could be avoided if low-cost storage systems could be made available. For these purposes, cryopreservation protocols based on the use of sucrose as a cryoprotectant in combination with encapsulation and evaporative dehydration would be particularly convenient. However, it is first necessary to assess the tolerance of the embryogenic tissues to high concentrations of sucrose and their ability to resist dehydration before testing the response to freezing procedures.

4.1 THE EFFECT OF DIFFERENT SUCROSE TREATMENTS ON THE SURVIVAL OF EMBRYOGENIC TISSUES FROM FIVE SWEET POTATO GENOTYPES

This experiment investigated the tolerance of embryogenic tissues of five sweet potato genotypes (865M, 1023M, 30MT, TIB10 and Papota) to elevated levels of sucrose. Eleven sucrose treatments (T_1-T_{11}) were tested for each genotype and the experimental procedure is described in Fig. 4.

The data presented in Table 4.1 show that viable embryogenic tissue was produced from three out five genotypes genotypes, following all of the sucrose treatments. However, there were significant differences between the responses of genotypes ($p<0.001$), and 1023M, 865M and TIB10 were the most responsive. Also, there were significant differences ($p<0.001$) between the effects of the eleven treatments of sucrose, with 0.4M sucrose (treatments T_5-T_7)

Table 4.1 Effect of sucrose treatments on the survival of embryogenic tissues from five sweet potato genotypes.

	Survival (%)									
	865M		1023M		30MT		TIB10		Papota	
Genotype	Embryo	Non-embryo	Embryo	Non-embryo	Embryo	Non-embryo	Embryo	Non-embryo	Embryo	Non-embryo
T_1	100	0	80	0	90	0	100	0	77	13
T_2	97	0	93	0	33	10	87	0	43	14
T_3	67	0	87	0	50	7	77	0	53	4
T_4	50	0	77	7	40	3	37	0	50	0
T_5	80	0	87	3	70	0	93	0	83	7
T_6	97	0	83	0	40	10	90	0	63	10
T_7	97	3	100	0	80	0	80	0	90	3
T_8	50	0	83	0	24	3	30	0	23	0
T_9	27	0	73	0	13	0	43	0	4	3
T_{10}	23	4	83	0	13	7	80	0	26	4
T_{11}	0	0	57	3	7	0	23	0	0	0

1 Sucrose treatments:

T_1: 0.1M(28d)
T_2: 0.1M(2d); →0.4M(2d) → 0.1M(28d)
T_3: 0.1M(2d); → 0.4M(2d); → 0.7M(2d) → 0.1M(28d)
T_4: 0.1M(2d); →0.4M(2d); →0.7M(2d); →1.0M(2d)→ 0.1M(28d)
T_5: 0.4M(1d)→ 0.1M(28d)
T_6: 0.4M(3d)→ 0.1M(28d)
T_7: 0.4M(5d)→ 0.1M(28d)
T_8: 0.7M(1d)→ 0.1M(28d)
T_9: 0.7M(3d)→ 0.1M(28d)
T_{10}: 0.7M(5d)→ 0.1M(28d)
T_{11}: 1.0M(1d)→ 0.1M(28d)

2. Basal medium:MS + 5μM 2,4-D

3. Replication: 3 petri dishes x 10 embryogenic aggregates (n=30)

4. Incubation conditions: 25°C / 16h photoperiod.

producing the better results with all of the genotypes, in terms of the embryogenic responses of surviving tissues.

4.2 THE EFFECT OF DIFFERENT SUCROSE TREATMENTS ON THE SURVIVAL OF ENCAPSULATED EMBRYOGENIC TISSUES FROM FOUR SWEET POTATO GENOTYPES

This experiment was designed to identify sucrose treatments which gave high survival rates for encapsulated embryogenic tissues (see Materials and Methods: section 2.6.2) from four sweet potato genotypes (865M, 1023M, 30MT and TIB10). Thirteen sucrose treatments (T_1-T_{13}) were tested with each genotype, and the experimental procedure is described in Fig. 5.

The data presented in Table 4.2 show that surviving encapsulated embryogenic tissue was produced from two of four genotypes following all of the sucrose treatments. However, there were significant differences between the responses of genotypes ($p<0.001$), and TIB10, 865M and 1023M were the most responsive. Also, there were significant differences ($p<0.001$) between the effects of the thirteen treatments of sucrose, with 0.4M sucrose (treatments T_7-T_9) producing the better embryogenic responses from surviving tissue with three of the genotypes.

4.3 THE EFFECT OF SUCROSE TREATMENT AND EVAPORATIVE DEHYDRATION ON THE SURVIVAL OF ENCAPSULATED EMBRYOGENIC TISSUES FROM SIX SWEET POTATO GENOTYPES

Following the results obtained in 4.2 showing the best survival of encapsulated embryogenic tissues cultured on media containing 0.4M sucrose, similar procedures were

Table 4.2 Effect of sucrose treatments on the survival of embryogenic tissues from four sweet potato genotypes.

	Survival (%)							
	865M		1023M		30MT		TIB10	
Genotype	Embryo	Non-embryo	Embryo	Non-embryo	Embryo	Non-embryo	Embryo	Non-embryo
T_1	60	13	76	24	37	16	80	0
T_2	77	10	50	43	20	33	73	3
T_3	67	13	70	27	13	14	67	0
T_4	63	30	30	57	3	27	20	43
T_5	60	37	20	67	0	23	43	17
T_6	40	30	3	7	0	27	13	47
T_7	60	10	73	14	47	20	87	3
T_8	57	13	73	20	43	0	83	4
T_9	77	13	64	33	60	3	70	0
T_{10}	17	26	20	10	10	53	33	0
T_{11}	7	6	4	3	0	10	30	0
T_{12}	0	3	10	7	0	3	27	0
T_{13}	0	0	20	7	0	23	7	0

n=30

1 Sucrose treatments:

T_1: 0.1M(28d)

T_2: 0.1M(3d) → 0.1M(28d)

T_3: 0.1M(5d) → 0.1M(28d)

T_4: 0.1M(7d) → 0.1M(28d)

T_5: 0.1M(10d)→ 0.1M(28d)

T_6: 0.1M(20d)→ 0.1M(28d)

T_7: 0.4M(1d)→ 0.1M(28d)

T_8: 0.4M(3d)→ 0.1M(28d)

T_9: 0.4M(5d)→ 0.1M(28d)

T_{10}: 0.7M(1d)→ 0.1M(28d)

T_{11}: 1.0M(3d)→ 0.1M(28d)

T_{12}: 0.7M(5d)→ 0.1M(28d)

T_{13}: 1.0M(1d)→ 0.1M(28d)

→ = de-bead

2 Basal medium:MS + 5μM 2,4-D

3 Replication: 3 petri dishes x 10 embryogenic aggregates (n=30)

4 Incubation conditions: 25°C / 16h photoperiod.

applied to six genotypes (865M, 1023M, 30MT, 209M, 132M and TIB10) with the addition of evaporative dehydration treatments. The full procedure is outlined in Fig. 6 and the survival data are recorded in Table 4.3.

The data in Table 4.3 show that there were significant differences between the responses of genotypes ($p<0.05$) and between the effects of dehydration times ($p<0.001$). Overall, the highest survival rates were obtained with dehydration times of up to 3h (24-41 moisture content), and with genotypes 209M and 132M for dehydration times of up to 5h (12-16% moisture content). With 6h dehydration, there was no survival of embryogenic tissues, although a small proportion of the aggregates from some of the genotypes survived only to produce non-embryogenic tissue subsequently.

4.4 THE EFFECT OF AN EXTERNAL SUCROSE TREATMENT AND EVAPORATIVE DEHYDRATION ON THE SURVIVAL OF ENCAPSULATED EMBRYOGENIC TISSUES FROM SIX SWEET POTATO GENOTYPES

The aim of this experiment was to establish whether the survival rates of embryogenic aggregates obtained in 4.3, following sucrose pre-treatment and evaporative dehydration could be further improved by the use of an extended pre-treatment period with a higher final sucrose concentration (0.7M).

The procedure was the same as that outlined in Fig. 6, except for the following changes:

i) Genotype 207M replaced genotype 132M

ii) The sucrose pre-treatment for the beads (Fig.6c) was modified to the following sequence: 0.4M(2d);0.7M(2d).

The data presented in Table 4.4 again show that there were significant differences between the responses of genotypes ($p<0.001$) and between the effects of the different

Table 4.3 Effect of sucrose treatment and evaporative dehydration on the survival of encapsulated embryogenic tissues from six sweet potato genotypes.

		Survival (%)											
		865M		1023M		30MT		209M		132M		TIB10	
Dehydration time (h)	Moisture content (%)	E	NE	E	NE	E	NE	E	NE	E	NE	E	NE
0	80-82	81	10	71	0	67	0	95	5	81	10	76	14
1	68-73	95	5	71	5	67	0	90	5	85	10	81	10
2	39-60	95	0	90	5	67	0	61	10	76	5	71	10
3	24-41	86	5	81	10	62	0	91	0	71	5	52	24
4	16-24	19	5	28	5	24	0	76	0	67	0	0	5
5	12-16	0	0	14	0	0	0	0	29	19	10	0	0
6	10-14	0	0	0	5	0	0	0	0	0	5	0	0

n=21

1. Sucrose treatment:

Before dehydration	After dehydration
0.1M(2d); encapsulation; 0.4M(3d)	0.1M(2d); debead; 0.1M(28d)

2. Basal Medium: MS + 5μM 2.4-D
3. Replication : 3 petridishes x 7 embryogenic aggregates (n=21)
4. Incubation conditions : 25°C /16h photoperiod (70μMm^{-2} s^{-1} PAR)
5. E = embryogenic, NE = non-embryogenic

dehydration times ($p<0.001$). In contrast with experiment 4.3, genotypes 865M and 1023M showed the highest survival rates after 5h dehydration (14-16% moisture content). However with all genotypes, improved survival rates were obtained with the longer dehydration times and except for one genotype (30MT), some embryogenic tissue was produced after 6h dehydration.

4.5 THE EFFECT OF SUCROSE TREATMENT, EVAPORATIVE DEHYDRATION AND MODIFIED POST-DEHYDRATION TREATMENT ON THE SURVIVAL OF ENCAPSULATED EMBRYOGENIC TISSUES FROM SWEET POTATO GENOTYPE 1023M

In this experiment, the protocol was modified, in comparison with that used in Fig. 6 to include a post-dehydration sequence in which the aggregates were taken through a series of gradually reducing sucrose concentration before the final incubation for 4 weeks on medium containing 0.1M sucrose.

The procedure was the same as that used in 4.3, except for the following changes.

i) A single genotype (1023M) was used.

ii) The sucrose pre-treatment for the beads (Fig. 6c) was modified to the following sequence: 0.4M(2d); 0.7M(2d).

iii) The post-dehydration treatment (Fig. 6) was modified to included the following sequence of sucrose treatments; beads transferred to 0.7M(1d);0.4M(1d); aggregates were removed from the beads; 0.4M(2d); 0.1M(28d).

The data in Table 4.5 showed significant differences ($p<0.05$) between the effects of the different dehydration times and the highest survival rates (100%) were obtained in the

Table 4.4 Effect of sucrose treatment and evaporative dehydration on the survival of encapsulated embryogenic tissues from six sweet potato genotypes.

		Survival (%)											
		865M		1023M		30MT		209M		207M		TIB10	
Dehydration time (h)	Moisture content (%)	E	NE	E	NE	E	NE	E	NE	E	NE	E	NE
0	67-72	85	10	71	5	33	53	95	0	62	14	81	14
1	53-61	81	10	81	0	52	15	81	5	38	14	71	10
2	31-41	57	14	48	0	48	28	76	0	38	24	76	19
3	23-31	71	0	100	0	71	14	81	10	62	14	71	24
4	17-22	95	0	80	6	19	0	52	0	38	38	48	28
5	14-16	81	0	76	0	10	4	19	0	47	24	14	19
6	9-14	29	0	42	10	0	0	48	10	14	0	3	0

n=21

1 Sucrose treatment:

Before dehydration	After dehydration
0.1M(2d); encapsulation; 0.4M(2d); 0.7M(2d)	0.1M(2d); debead; 0.1M(28d)

2 Basal Medium: MS + 5μM 2.4-D

3 Replication : 3 petridishes x 7 embryogenic aggregates (n=21)

4 Incubation conditions : 25°C /16h photoperiod ($70Mm^{-2}S^{-1}$ PAR)

5 E = embryogenic, NE = non-embryogenic

Table 4.5 Effect of sucrose treatment and evaporative dehydration on the survival of encapsulated embryogenic tissues in genotype 1023M

		Survival (%)	
Dehydration time (h)	Moisture content (%)	Embryogenic	Non-Embryogenic
0	70	90	5
1	59	90	5
2	41	100	0
3	29	100	0
4	22	100	0
5	18	100	0
6	15	71	0

n=21

1 Sucrose treatment:

Before dehydration	After dehydration
0.1M(3d); encapsulation; 0.4M(3d); 0.7M(2d)	0.7M(1d); 0.4M(1d); debead; 0.4M(2d); 0.1M(28d)

2 Basal Medium: MS + 5µM 2.4-D

3 Replication : 3 petridishes x 7 embryogenic aggregates (n=21)

4 Incubation conditions : 25°C /16h photoperiod ($70Mm^{-2} S^{-1}$ PAR)

intermediate range of 2-5h. In comparison with the response of genotype 1023M in 4.4, higher overall survival rates were obtained.

4.6 THE EFFECT OF SUCROSE TREATMENT, EVAPORATIVE DEHYDRATION AND A TWO-STEP FREEZING PROCEDURE ON THE SURVIVAL OF ENCAPSULATED EMBRYOGENIC TISSUES IN THE SWEET POTATO GENOTYPE 1023M

Following the studies with sucrose treatments and evaporative dehydration in 4.1 to 4.5, the effects of a protocol that included a two-step freezing procedure were investigated with genotype 1023M. The procedure was similar to that outlined in Fig. 6, with the insertion of a freezing/thawing sequence following dehydration (see Fig. 7 for details). The data presented in Table 4.6 indicate that there were significant differences between the effects of the different dehydration times on survival of the frozen aggregates ($p < 0.001$). The highest embryogenic response was obtained after 4h dehydration (14-16% moisture content). Compared with the non-frozen controls, the overall survival rates of the frozen tissues were somewhat lower, but considerably higher proportions produced non-embryogenic tissue. With the non-frozen controls, the differences between the effects of the different dehydration times were significant, and overall, the survival rates were a little lower than those obtained with the same genotype in 4.5, when slightly different sucrose treatments were employed.

Table 4.6 Effect of sucrose treatment, evaporative dehydration and two-step freezing on the survival of encapsulated embryogenic tissues in genotype 1023M

Dehydration (h)	Moisture content(%)	Survival (%)			
		Non-frozen		Two-step freezing	
		E	NE	E	NE
3	18-21	95	0	24	52
4	14-16	86	5	62	29
5	13-14	71	5	38	24

n=21

1 Sucrose treatment:

Before dehydration/freezing: 0.1M(3d); encapsulation; 0.4M(2d); 0.7M(2d)

After dehydration/freezing: 0.1M(2d); debead; 0.1M(28d)

2 Basal Medium: MS + 5μM 2.4-D

3 Replication : 3 petridishes x 7 embryogenic aggregates (n=21)

4 Incubation conditions : 25°C /16h photoperiod ($70Mm^{-1}$ S^{-1} PAR), immediately after thawing, all cultures were incubated in the dark at 25°C for 1d

5 E = embryogenic, NE = non-embryogenic

6 Freezing procedure: ambient temperature to 0°C at $10min^{-1}$; 0°C to –40°C at 0.5°C, followed by transfer to LN_2

4.7 THE EFFECT OF SUCROSE, EVAPORATIVE DEHYDRATION AND A TWO STEP FREEZING PROCEDURE ON THE SURVIVAL OF ENCAPSULATED EMBRYOGENIC TISSUES FROM EIGHT SWEET POTATO GENOTYPES

Because of the number of treatments (five sucrose pre and post-freezing treatments, and three dehydration times) investigated with each of eight genotypes, it was necessary to deal with the individual genotypes in separate experiments. Identical procedures based on the results obtained in 4.1-4.6 were therefore applied to the eight genotypes in a series of experiments (4.7.1-4.7.8) over a period of 16 weeks. As far as comparison between the genotypes are concerned, this is not an ideal arrangement because of the difficulties in ensuring that the various genotypes are in a similar physiological state for each experiment. This approach was unavoidable, and no doubt this contributed to some of the observed differences between the responses of genotypes. The preliminary aim, however, was to identify robust procedures which could be used with a wide range of genotypes without the need to use highly specified physiological states, requiring extensive preliminary work before cryopreservation could be applied.

For each genotype, the experimental procedure was similar to that outlined in Fig. 7 with the following modifications:

i) 30 experimental interactions were employed (5 pre-freezing/post-freezing protocols x 3 dehydration times x frozen and unfrozen treatments).

ii) The different sucrose treatments involved the following sequences before (replacing Fig 7 a-c) and after (replacing Fig. 7 g-j) dehydration/freezing and thawing:

Table 4.7: Effect of sucrose treatment, evaporative dehydration and two-step freezing on the survival of encapsulated somatic embryogenic tissue in genotype 865M.

			Survival %			
			Non-frozen		Two-step freezing	
Sucrose Treatment	Dehydration time (hrs)	Moisture contents (%)	Embryogenic	Non-embryogenic	Embryogenic	Non-embryogenic
T_1	3	25-37	81	0	62	0
	4	23-31	76	0	48	0
	5	17-27	87	0	71	0
T_2	3	17-18	95	0	38	0
	4	16-17	81	0	29	0
	5	15	43	0	5	0
T_3	3	16-18	86	0	19	0
	4	13-14	57	0	0	0
	5	12-14	19	0	0	0
T_4	3	13	38	10	33	0
	4	13	29	0	14	0
	5	12-13	14	0	0	0
T_5	3	20	29	4	10	5
	4	17-18	10	0	5	5
	5	16-17	38	17	24	9

n = 21

1 Sucrose Treatments:

	Before freezing	*After freezing/thawing*
T1:	0.1M(3d); Encapsulation: 0.4M (3d); 0.7M(2d)	0.1M(2d);de-bead;0.1M(28d)
T2:	0.1M(3d); Encapsulation: 0.4M(3d);0.7M(2d);1.0M(2d)	0.1M(2d);de-bead;0.1M(28d)
T3:	0.1M(3d); Encapsulation: 0.4M (3d); 0.7M(2d)	0.7M(1d);0.4M(1d);de-bead;0.4M(2d);0.1M(28d)
T4:	0.1M(3d); Encapsulation: 0.4M (3d); 0.7M(2d)	0.4M(2d);d-bead;0.4M(1d);0.1M(28d)
T5:	0.1M(3d); Encapsulation: 0.4M (5d); 0.7M(3d)	0.1M(2d);de-bead;0.1M(28d)

2 Basal Medium: MS+ 5µM 2,4-D
3 Replication: 3 petri dishes x 7 embryogenic aggregates (n=21)
4 Incubation Conditions: 25°C/16h photoperiod. Immediately after thawing, all cultures were incubated in the dark at 25°C for 1d.
5 Freezing procedure: ambient temperature to 0°C at $10min^{-1}$; 0°C to -40 °C at 0.5 °C min^{-1}; followed by transfer to liquid nitrogen.

Before dehydration/ freezing	After freezing/thawing
T_1:0.1M(3d): encapsulation: 0.4M(3d):0.7M(2d)	0.1M(2d): de-bead: 0.1M(28d)
T_2 :0.1M(3d): encapsulation: 0.4M(3d):0.7M(2d):1.0M(2d)	0.1M(2d): de-bead:0.1M(28d)
T_3 : 0.1M(3d): encapsulation: 0.4M(3d):0.7M(2d)	0.7M(1d)0.4M(1d): de-bead: 0.1M(28d)
T_4 : 0.1M(3d): encapsulation: 0.4M(3d):0.7M(2d)	0.4M(2d): de-bead: 0.4M(1d): 0.1M(28d)
T_5 : 0.1M(3d): encapsulation: 0.4M(5d):0.7M(3d)	0.1M(2d): de-bead: 0.1M(28d)

For each genotype and sucrose treatments, the data is presentd for survival of freezing after dehydration times of 3,4 and 5 hours. The moisture contents after 3 hours ranged from 19-34%, after 4 hours from 14-31% and after 5 hours from 11-27%.

4.7.1 GENOTYPE 865M

The data presented in Table 4.7 indicate that the T_1 sucrose treatment produced significantly higher ($p<0.05$) survival rates (mean=60%) for frozen embryogenic tissues than any other sucrose treatments. The survival rates for the different dehydration times were not significantly different from each other, although the higher values were obtained with the 4h period (mean=32%).

4.7.2 GENOTYPE 1023M

The data presented in Table 4.8 and the analysis of variance (not shown) indicate that the T_1 and T_3 sucrose treatments produced significantly higher ($p<0.01$) survival rates, (means=38% and 32% respectively), of embryogenic tissues following freezing than the T_2 and T_5 treatments. The survival rates with the 4h and 5h dehydration times (means=27% and 29% respectively) were significantly higher ($p<0.01$) than those obtained with the 3h dehydration times (mean = 4%).

4.7.3 GENOTYPE 30MT

The data presented in Table 4.9 indicate that in general, the embryogenic tissues responded well to the cryopreservation procedures, with relatively high survival rates being obtained with many treatments as well as with the controls. Overall, the T_2 sucrose treatment (mean=65%) was the most successful with regard to the survival of frozen embryogenic tissues, but the results were only significantly better ($p<0.01$) than the T_1 and T_3 treatments (means=14% and 37% respectively). The 4h dehydration times produced the highest survival rates (mean=48%), but they were not significantly better than those obtained with the 3 and 5h dehydration times (means=44% and 30% respectively).

4.7.4 GENOTYPE 209M

The data presented in Table 4.10 indicate that, in general, relatively high survival rates were obtained with both the frozen tissues and the controls. Even higher survival rates were obtained if account is taken of the relatively high proportion of tissues that survived, despite losing their embryogenic competence. Overall, the T_4 sucrose treatment produced high survival rates (mean=48%) with the frozen embryogenic tissues, but the results were only significantly better ($p<0.01$) than those obtained with the T_1 and T_2 treatments (means=18% and 19% respectively). The 3h dehydration time produced the highest survival rates for the frozen tissues (mean=42%), but the values were not significantly better than those obtained with 4h and 5h (means=32% and 26% respectively).

4.7.5. GENOTYPE 207M

The data presented in Table 4.11 indicate that, as with genotype 209M, the production of non-embryogenic tissue, both in controls and following the treatments involving

Table 4.8: Effect of sucrose treatments, evaporative dehydration and two-step freezing on the survival of encapsulated somatic embryos in genotype 1023M.

			Survival %			
			Non-frozen		Two-step freezing	
Sucrose Treatment	Dehydration time (hrs)	Moisture contents (%)	Embryogenic	Non-embryogenic	Embryogenic	Non-embryogenic
T_1	3	23-31	67	0	14	0
	4	19-20	71	0	33	19
	5	16-18	82	5	67	23
T_2	3	16-19	86	9	5	24
	4	14-17	67	14	5	9
	5	14	24	14	0	0
T_3	3	18-28	76	24	0	0
	4	18-23	95	5	67	0
	5	15-17	90	0	29	9
T_4	3	17-32	90	5	0	0
	4	16-21	48	14	19	5
	5	17	70	20	38	14
T_5	3	18-21	81	5	0	0
	4	15-17	81	14	0	0
	5	13-15	62	9	0	0

n=21

For experimental details see Table 4.7.

Table 4.9: Effect of sucrose treatments, evaporative dehydration and two-step freezing on the survival of encapsulated somatic embryos in genotype 30MT.

			Survival %			
			Non-frozen		Two-step freezing	
Sucrose Treatment	Dehydration (hrs)	Moisture contents (%)	Embryogenic	Non-embryogenic	Embryogenic	Non-embryogenic
T_1	3	19-29	57	0	19	0
	4	18-28	67	0	14	5
	5	17-23	52	0	10	14
T_2	3	27-30	71	0	67	0
	4	26-27	85	5	76	0
	5	24	76	0	52	0
T_3	3	28-35	86	0	57	0
	4	27-29	76	0	43	5
	5	24-25	62	0	10	0
T_4	3	27-36	67	19	33	5
	4	26-32	57	0	57	5
	5	24-28	67	0	33	0
T_5	3	31-41	81	14	43	0
	4	30-34	81	5	52	0
	5	28-33	76	0	52	0

n=21

For experimental details see Table 4.7.

Table 4.10: Effect of sucrose treatments, evaporative dehydration and two-step freezing on the survival of encapsulated somatic embryogenic tissue in genotype 209M.

			Survival %			
			Non-frozen		Two-step freezing	
Sucrose Treatment	Dehydration time (hrs)	Moisture contents (%)	Embryogenic	Non-embryogenic	Embryogenic	Non-embryogenic
T_1	3	28-32	57	34	29	28
	4	23-29	81	14	10	4
	5	20-27	52	24	14	5
T_2	3	22-30	62	9	24	5
	4	22-29	48	0	14	24
	5	20-25	38	10	19	19
T_3	3	26-33	71	24	47	5
	4	25-27	90	0	57	0
	5	21-24	57	0	33	0
T_4	3	24-33	100	0	67	9
	4	21-33	90	5	38	24
	5	20-23	81	9	38	38
T_5	3	27-38	81	19	43	14
	4	24-32	90	10	43	14
	5	22-24	67	19	24	28

n=21

For experimental details see Table 4.7.

Table 4.11: Effect of sucrose treatments, evaporative dehydration and two-step freezing on the survival of encapsulated embryogenic tissue in genotype 207M.

			Survival %			
			Non-frozen		Two-step freezing	
Sucrose Treatment	Dehydration time (hrs)	Moisture contents (%)	Embryogenic	Non-embryogenic	Embryogenic	Non-embryogenic
T_1	3	33-36	14	24	0	19
	4	25-30	19	29	0	10
	5	24-27	0	0	0	0
T_2	3	26-32	67	24	0	0
	4	23-24	76	24	10	29
	5	23	76	14	43	29
T_3	3	18-21	67	9	43	9
	4	18	57	19	29	14
	5	14-15	29	14	0	19
T_4	3	18-25	86	4	14	5
	4	16-19	57	0	38	19
	5	16	38	14	29	19
T_5	3	25-27	67	23	33	24
	4	23-27	52	38	29	48
	5	22-26	38	42	19	43

n=21

For experimental details see Table 4.7.

Table 4.12: Effect of sucrose treatments, evaporative dehydration and two-step freezing on the survival of encapsulated embryogenic tissue in genotype 132M.

			Survival %			
			Non-frozen		Two-step freezing	
Sucrose Treatment	Dehydration time (hrs)	Moisture contents (%)	Embryogenic	Non-embryogenic	Embryogenic	Non-embryogenic
T_1	3	24-31	67	0	29	20
	4	23-24	38	5	5	0
	5	20-22	33	0	0	0
T_2	3	20-33	71	10	33	14
	4	17-21	48	19	29	9
	5	17-18	52	5	14	10
T_3	3	22-30	29	28	0	10
	4	19-23	19	19	0	5
	5	17-22	10	9	0	5
T_4	3	25-33	67	14	0	0
	4	21-28	43	14	0	0
	5	18-21	0	0	0	0
T_5	3	24-34	81	5	28	10
	4	22-25	52	10	33	14
	5	21-22	43	19	14	14

n=21

For experimental details see Table 4.7.

freezing was relatively high. Overall, there was relatively little difference between the effects of the various freezing treatments, except for sucrose treatment T_1 following which there was no survival. The remaining sucrose treatments, T_2 to T_5 (means=18%, 24%, 27% and 27% respectively), and the three dehydration times (3 to 5h) (means=19%, 22% and 19% respectively) did not produce significantly different results.

4.7.6. GENOTYPE 132M

A notable feature of the results obtained in Table 4.12 with both the frozen and non-frozen tissues from the genotype (see 4.7.6) was the relatively low overall survival rates. Two of the sucrose treatments (T_2 and T_5) produced significantly higher ($p<0.001$) survival rates with the frozen tissues (means=25% and 25% respectively), whereas there was no survival with a further two of the treatments (T_3 and T_4). The 3h dehydration time produced the highest survival rates with the frozen tissue (mean=17%), but the results were only significantly higher than those obtained with the 5h dehydration.

4.7.7. GENOTYPE TIB10

The data presented in Table 4.13 indicate that the sucrose treatment T_1 (mean=56%) produced significantly higher survival rates ($p<0.05$) with the frozen tissues than the T_2 and T_5 treatments (means=8% and 24% respectively). The 4h dehydration time produced the highest survival rates (mean=37%), but these were only significantly higher than the rates obtained with 5h dehydration (mean=16%).

4.7.8. GENOTYPE PAPOTA

The data presented in Table 4.14 show that, as with genotype 132M, low overall survival rates were obtained with both frozen and non-frozen tissues. Although, the mean survival

Table 4.13: Effect of sucrose treatments, evaporative dehydration and two-step freezing on the survival of encapsulated embryogenic tissue in genotype TIB10.

			Survival %			
			Non-frozen		Two-step freezing	
Sucrose Treatment	Dehydration time (hrs)	Moisture contents (%)	Embryogenic	Non-embryogenic	Embryogenic	Non-embryogenic
T_1	3	23-33	67	5	57	5
	4	22-28	95	0	71	0
	5	20-24	71	5	38	9
T_2	3	23-25	24	14	19	5
	4	22-24	5	33	0	0
	5	21-23	19	24	5	5
T_3	3	31-34	67	5	5	0
	4	29-33	52	10	43	0
	5	27-30	43	10	24	0
T_4	3	31-36	71	14	19	0
	4	28-34	90	10	48	10
	5	27-32	33	5	5	0
T_5	3	28	62	5	14	5
	4	23-24	48	14	5	19
	5	21-22	24	10	5	0

n=21

For experimental details see Table 4.7.

rate obtained with T_3 treatment (mean=8%) were the highest among the results obtained with various sucrose treatments. The results were not significantly better than those obtained with any of the others. Likewise, the results obtained with the 4h dehydration time (mean=2%), although highest, were not significantly higher than the results from the other treatments.

4.7.9. GENERAL CONCLUSION FROM EXPERIMENTS 4.7.1-4.7.8

1 There was some survival of embryogenic tissue from all genotypes following the freezing procedure with all of the genotypes ranging between means of 65% (genotype 30MT) and 8% (genotype Papota)

2 There was no consistent pattern of successful treatments among the various genotypes, although the T_1 sucrose treatment and the 4h dehydration times produced some of the best results with frozen tissues.

3 The highest overall survival rates with frozen tissues were obtained with genotypes 30MT and 865M (means=65% and 60% respectively) and the lowest survival rates were obtained with genotypes 132M and Papota (means=25% and 8% respectively).

4 Generally, the high survival rates for the frozen tissues were associated with high survival rates among the non-frozen controls.

5 Certain genotypes (particularly 209M and 207M) were characterised by a relatively high loss of embryogenic competence among surviving frozen tissues.

Table 4.14: Effect of sucrose treatments, evaporative dehydration and two-step freezing on the survival of encapsulated embryogenic tissue in genotype Papota.

			Survival %			
			Non-frozen		Two-step freezing	
Sucrose Treatment	Dehydration time (hrs)	Moisture contents (%)	Embryogenic	Non-embryogenic	Embryogenic	Non-embryogenic
T_1	3	30-34	57	10	0	10
	4	29-33	0	0	0	0
	5	24-28	0	0	0	0
T_2	3	30-31	76	19	50	0
	4	27	33	5	10	0
	5	23-25	20	0	10	4
T_3	3	26-28	38	5	5	0
	4	21-24	57	0	14	0
	5	14-19	38	0	5	0
T_4	3	19-36	38	0	0	0
	4	18-21	38	0	0	0
	5	16-19	57	0	10	0
T_5	3	38-41	33	5	0	0
	4	29-30	14	23	0	5
	5	28-29	19	5	5	5

n=21

For experimental details see Table 4.7.

4.8 THE EFFECT OF SUCROSE EVAPORATIVE DEHYDRATION PROTOCOL ON THE SURVIVAL OF ENCAPSULATED EMBRYOGENIC TISSUES IN SIX SWEET POTATO GENOTYPES

Following the results obtained in experiments 4.7.1-4.7.8; where it had been necessary to subject genotypes separately to a series of cryopreservation protocols involving different sucrose and dehydration treatments, a further experiment was carried out in which a slightly modified version of one of the more successful protocols was applied to six genotypes in a single experiment to improve the survival rate of embryogenic tissues. The experimental procedures were similar to those used in treatments T_1 and T_5 in the previous series of experiments (see Fig. 7 and Section 4.7 for details) with minor changes in the durations of the stages for the sucrose pre-freezing treatment:

Before dehydration/ freezing	After freezing/ thawing
0.1M(4d), encapsulation; 0.4M (3d); 0.7M(3d)	0.1M(2d), debead; 0.1M(28d)

The data presented in table 4.15 indicate that the genotypes 865M, 207M and 209M showed higher survival rates for frozen embryogenic tissues than any other genotypes. With non-frozen controls, there were no significant differences between the dehydration times, but the 3h dehydration time produced higher survival rates of embryogenic tissues. These results are only partly consistent with the results obtained in the previous series of experiments 4.7.1-4.7.8) in that genotype 865M produced the highest survival rates for frozen embryogenic tissues with sucrose treatment T_1. In addition, genotypes 207M and 209M again showed a significant proportion of the surviving tissues to be non-embryogenic. Strictly, however, direct comparison cannot be made between the experiments because slightly different sucrose treatments were employed.

Table 4.15: Effect of sucrose treatments, evaporative dehydration and two-step freezing on the survival of encapsulated embryogenic tissue in six sweet potato genotypes

			Survival %			
			Non-frozen		Two-step freezing	
Genotype	Dehydration (hrs)	Moisture contents (%)	Embryogenic	Non-embryogenic	Embryogenic	Non-embryogenic
865M	0	72-73	100	0	0	0
	3	21-23	52	10	29	0
	4	20-22	52	0	24	0
	5	19-21	24	0	0	0
1023M	0	72-73	100	0	0	0
	3	18-20	62	9	5	0
	4	18-20	33	10	0	0
	5	17-19	5	0	0	0
30MT	0	69-72	95	5	0	0
	3	23-24	19	62	5	0
	4	21-23	33	5	0	0
	5	20-21	0	0	0	0
209M	0	73	100	0	0	0
	3	21-22	81	5	24	5
	4	19-21	86	10	19	19
	5	19-20	19	19	0	0
207M	0	72-73	90	10	0	0
	3	25	81	14	43	14
	4	23-25	48	29	0	5
	5	20-24	29	43	0	10
132M	0	73-74	95	0	0	0
	3	28	48	14	0	0
	4	25-27	10	9	0	0
	5	24	5	9	0	0

1 Sucrose treatment:

Before Dehydration	After dehydration
0.1M(4d); encapsulation; 0.4M(3d); 0.7M(3d)	0.1M(2d); debead; 0.1M(28d)

2 Basal Medium: MS + 5μM 2.4-D

3 Replication : 3 petridishes x 7 embryogenic aggregates (n=21)

4 Incubation conditions : 25°C /16h photoperiod (70μMm^{-2} s^{-1} PAR), immediately after thawing, all cultures were incubated in the dark at 25°C for 1d

4.9 THE EFFECTS OF SUCROSE EVAPORATIVE-DEHYDRATION PROTOCOLS ON THE SURVIVAL OF ENCAPSULATED EMBRYOGENIC TISSUES IN A SERIES OF REPEATED EXPERIMENTS WITH FOUR SWEET POTATO GENOTYPES

Comparing the results obtained in experiments 4.7.1-4.7.8 with those in experiment 4.8, it would seem that the broad trends are reproducible in the sense that two-step freezing in combination with suitable sucrose treatments and evaporative dehydration can provide the basis for reasonably successful cryopreservation protocols for use with sweet potato embryogenic tissues. More detailed comparison between the effects of particular protocols on individual genotypes, however, show that precise quantitative responses were not reproduced, with the result that it would not, on the basis of these results, be possible with any confidence to rank the protocols in terms of effectiveness. It was decided, therefore, to make a further series of comparisons involving four genotypes, each subjected to one or two of the protocols employed in experiments 4.7.1-4.7.8.

The basic experimental procedure was similar to that employed in 4.7.1-4.7.8 (see Fig. 7 and section 4.7 for details), omitting non-frozen treatments, and the one or two sucrose treatments selected for investigation with each genotype are indicated below, using the same codes that were employed in the earlier experiments.

4.9.1 GENOTYPE 30MT

Sucrose treatments T2 and T5 were selected for investigation because they produced the highest survival rates in experiment 4.7.3. The data presented in Table 4.16 indicate that, overall, the T2 treatment produced higher survival rates than the T5 treatment following two-step freezing (mean=30% compared with mean = 25%).

Table 4.16 Effect of sucrose treatment, evaporative dehydration and two-step freezing on the survival of encapsulated embryogenic tissues in genotype 30MT

Sucrose treatment	Dehydration time (h)	Moisture content (%)	Survival (%)	
			Two-step freezing	
			Embryogenic	Non-embryogenic
T_2	3	25	33	0
	4	24	0	5
	5	22	57	10
T_5	3	28	33	0
	4	24	28	0
	5	22	14	0

n=21

1 Sucrose treatment:

	Before Dehydration / freezing	After dehydration / thawing
T_2	0.1M(3d): encapsulation: 0.4M(3d): 0.7M(2d): 1.0M (2d)	0.1M(2d): de-bead: 0.1M (28d)
T_5	0.1M(3d): encapsulation: 0.4M(2d): 0.7M(3d)	0.1M(2d): de-bead: 0.1M (28d)

2 Basal Medium: MS + 5μM 2,4-D

3 Replication: 3 Petri dishes x 7 embryogenic aggregates (n=21)

4 Incubation conditions : 25°C /16h photoperiod (70μM m^{-2} s^{-1} PAR), immediately after thawing, all cultures were incubated in the dark at 25°C for 1d

5 Freezing procedure: ambient temperature to 0°C at 10 min^{-1}; 0°C to –40°C at 0.5°C min^{-1}, followed by transfer to LN_2 for 1h.

Table 4.17 Effect of sucrose treatment, evaporative dehydration and two-step freezing on the survival of encapsulated embryogenic tissues in genotype TIB10

Sucrose treatment	Dehydration time (h)	Moisture content (%)	Survival(%)	
			Two-step freezing	
			Embryogenic	Non-embryogenic
T_1	3	27	38	19
	4	25	57	14
	5	24	29	10
T_5	3	21	14	0
	4	20	0	5
	5	20	0	0

n=21

1 Sucrose treatment:

	Before dehydration / freezing	After dehydration / thawing
T_1	0.1M(3d): encapsulation: 0.4M(3d): 0.7M(2d)	0.1M(2d): de-bead: 0.1M (28d)
T_5	0.1M(3d): encapsulation: 0.4M(5d): 0.7M(3d)	0.1M(2d): de-bead: 0.1M (28d)

For further experimental details see Table 4.16

4.9.2 GENOTYPE TIB10

Sucrose treatments T_1 and T_5 were selected for investigation, because they produced the highest and lowest survival rates in experiment 4.7.7. The data presented in Table 4.17 indicate that the overall survival rates with treatment T_1 were higher in experiment 4.7.7 (mean =56% compared with mean = 41%) and slightly lower with treatment T_5 (mean=24%, compared with mean = 5%). In both experiments, treatment T_1 gave better overall results than treatment T_5 with the 4h dehydration period (25% moisture content) producing the highest survival rate, in contrast to treatment T_5 where the 3h dehydration period was optimal.

4.9.3 GENOTYPE 1023M

Sucrose treatments T_1 and T_5 were selected for investigation because they produced the highest and lowest survival rates in experiment 4.7.2. The data presented in Table 4.18 indicate that the overall survival rates with treatment T_1 were considerably higher in experiment 4.7.2 (mean=38% compared with mean = 19%), but in both experiments survival increased as the dehydration time was extended from 3h to 5h (29% to 26% moisture content). Also, in both experiments, survival was very low with treatment T_5.

4.9.4 GENOTYPE 207M

Sucrose treatment T_1 was selected for investigation because it produced lower survival rates in experiment 4.7.5. The data presented in table 4.19 indicate that overall survival rates although fairly low (mean = 10%) were actually higher than those obtained in experiment 4.7.5 where there was no survival of embryogenic tissue. In both experiments with this genotype, there was a tendency for a relatively high survival of tissue producing a non-embryogenic response.

Table 4.18 Effect of sucrose treatment, evaporative dehydration and two-step freezing on the survival of encapsulated embryogenic tissues in genotype 1023M

Sucrose treatment	Dehydration time (h)	Moisture content (%)	Survival(%)	
			Two-step freezing	
			Embryogenic	Non-embryogenic
T_1	3	29	5	24
	4	27	19	14
	5	26	33	29
T_5	3	24	10	19
	4	21	0	5
	5	20	0	5

n=21

1 Sucrose treatment:

	Before dehydration / freezing	After dehydration / thawing
T_1	0.1M(3d); encapsulation; 0.4M(3d); 0.7M(2d)	0.1M(2d); de-bead; 0.1M (28d)
T_5	0.1M(3d); encapsulation; 0.4M(5d); 0.7M(3d)	0.1M(2d); de-bead; 0.1M (28d)

For further experimental details see Table 4.16

Table 4.19 Effect of sucrose treatment, evaporative dehydration and two-step freezing on the survival of encapsulated embryogenic tissues in genotype 207M

Sucrose treatment	Dehydration time (h)	Moisture content (%)	Survival(%)			
			Non-frozen		Two-step freezing	
			Embryogenic	Non-embryogenic	Embryogenic	Non-embryogenic
T_1	3	29	57	14	24	33
	4	27-28	43	29	5	29
	5	25-27	24	33	0	9

n=21

1 Sucrose treatment:

Before Dehydration / freezing	After dehydration / thawing
T_1: 0.1M(3d); encapsulation; 0.4M(3d); 0.7M(2d)	0.1M(2d); de-bead; 0.1M (28d)

For further experimental details see Table 4.16

4.9.5 General conclusion from experiments 4.9.1-4.9.4

Although broadly similar trends were observed in the responses of genotypes to similar cryopreservation treatments in experiments 4.7 and 4.9, there were some inconsistencies. In particular, there were quite large differences in the individual survival rates recorded in the equivalent experiments.

4.10 THE EFFECT OF CULTURE AGE, EVAPORATIVE DEHYDRATION AND A TWO-STEP FREEZING PROCEDURE ON THE SURVIVAL OF ENCAPSULATED EMBRYOGENIC TISSUES FROM THE SWEET POTATO GENOTYPES

It was evident from the results in the series of experiments in section 4.7 to 4.9 that some success had been achieved in the development of a cryopreservation procedure that could be used with embryogenic tissue from a range of sweet potato genotypes. It was, however, also evident that in quantitative terms the results were rather erratic between experiments; for practical purposes therefore, further improvements should be made. One possibility was that the uneven results were, at least partially, caused by differences in the physiological status of the tissues used in different experiments. This was because it had not been possible, for practical reasons, to ensure that the tissues were always used at exactly the same stage in the culture cycle. To investigate this possibility, experiments were carries out with two genotypes (865M and 30MT), in which embryogenic tissues taken at different times following subculture were subjected to a standard cryopreservation protocol based on one of those employed in the previous experiments. The basic experimental procedure was similar to that used in 4.7.1-4.7.8 (see Fig 7 and

Section 4.7 for details), employing sucrose treatment T_1. The embryogenic tissues were taken for investigation 7, 14, 21 and 28d after subculture.

4.10.1 GENOTYPE 865M

The data in Table 4.20 indicate that there were significant differences ($p< 0.05$) between the responses of the tissues of different ages, with the 28d tissues showing the highest survival rates among the frozen tissues. No significant differences between dehydration times were recorded. With non-frozen controls, only the 3h dehydration time (32-38% moisture content) produced significantly higher survival rates ($p< 0.1$) of embryogenic tissues. A significant difference between ($p< 0.001$) the responses of the tissue of different ages, with the 14d and 28d tissues showing the highest survival rates among non-frozen tissue.

4.10.2 GENOTYPE 30MT

The data presented in Table 4.21 show that there were significant differences ($p< 0.001$) between the responses of the tissues of different ages, with the 14d and 28d tissue showing the highest survival rates among the frozen tissues. There was no significant effect of dehydration (24-26% moisture contents) but overall the 5h dehydration produced higher survival rates for the frozen tissues. With non-frozen controls, 3h dehydration time produced significantly higher survival rates ($p<0.001$). There were significant differences ($p<0.05$) between the responses of the tissues of different ages, with 14d and 28d tissue showing the higher survival rates among non-frozen controls.

Table 4.20: Effect of culture age, evaporative dehydration and two-step freezing on the survival of encapsulated embryogenic tissues in genotype 865M.

			Survival %			
			Non-frozen		Two-step freezing	
Age of culture (d)	Dehydration time (hr)	Moisture contents (%)	Embryogenic	Non-embryogenic	Embryogenic	Non-embryogenic
7	3	28-30	39	25	14	10
	4	24-26	32	21	18	21
	5	22	18	25	0	14
14	3	33-36	93	4	0	0
	4	25-26	79	0	7	0
	5	22-25	61	0	36	0
21	3	23-26	89	7	21	0
	4	20-22	62	14	14	11
	5	17-18	52	14	7	0
28	3	32-38	93	0	7	0
	4	24-27	79	11	39	21
	5	22-24	54	14	36	7

n=28

1 Pre-culture treatment:

Before dehydration / freezing — 0.1M(3d); Encapsulation: 0.4M (3d); 0.7M(2d)

After freezing/thawing — 0.1M(2d);de-bead;0.1M(28d)

2 Freezing procedure: ambient temperature to 0°C at $10min^{-1}$; 0°C to -40 °C at 0.5 °C min-^{1}; followed by transfer to LN_2 for 1h.

3 Basal Medium: MS+ 5μM 2,4-D

4 Replication: 4 petri dishes x 7 embryogenic aggregates (n=28)

5 Incubation Conditions: 25°C/16h photoperiod ($70\mu Mm^{-2}s^{-1}$ PAR). Immediately after thawing, all cultures were incubated in the dark at 25°C for 1d.

Table 4.21: Effect of culture age, evaporative dehydration and two-step freezing on the survival of encapsulated embryogenic tissue in genotype 30MT.

			Survival %			
			Non-frozen		Two-step freezing	
Age of culture (d)	Dehydration time (hrs)	Moisture contents (%)	Embryogenic	Non-embryogenic	Embryogenic	Non-embryogenic
7	3	21-26	43	14	0	0
	4	20-21	43	0	7	4
	5	19	7	7	14	0
14	3	34	64	0	18	0
	4	28	25	0	7	4
	5	24-26	14	0	18	7
21	3	28-31	33	14	0	0
	4	26-29	19	5	4	0
	5	25-26	7	5	0	0
28	3	26-32	64	25	7	11
	4	24-28	43	29	14	18
	5	24-26	14	43	18	18

n=28

For experimental details see Table 4.20

4.11 SURVIVAL OF CRYOPRESERVED EMBRYOGENIC TISSUE FROM TWO SWEET POTATO GENOTYPES DURING EXTENDED STORAGE PERIODS IN LIQUID NITROGEN

Although it is often stated on theoretical grounds that there should be no deterioration in tissue stored at the temperature of liquid nitrogen, it is important that this assumption is supported by empirical evidence. Two genotypes (865M and 209M) were therefore subjected to the optimal cryopreservation protocols identified in experiment 4.7, and then stored in liquid nitrogen for periods of up to 120d before thawing and assessment of viability.

The experimental procedure was similar to that employed in 4.7.1-4.7.8 (see Fig.7 and Section 4.7 for details), omitting the non-frozen controls. The T_1 and T_4 sucrose treatments were selected for genotypes 865M and 209M respectively, and after freezing, the tissues were stored in liquid nitrogen for periods of 30, 60, 90 and 120d.

The data presented in Table 4.22 show that there were no significant effects of the extended storage treatments on the survival of frozen tissues from either of the genotypes. The data also show that there were significant differences ($p<0.01$) between the effects of the dehydration times on survival rates, with 3h (25-31% moisture content) producing the highest values for both genotypes.

4.12 THE EFFECT OF ENCAPSULATION MEDIA, EVAPORATION DEHYDRATION AND RAPID FREZING ON THE SURVIVAL OF ENCAPSULATED EMBRYOGENIC TISSUES IN GENOTYPE 865M

The rapid cooling method, accomplished by the direct immersion of the cryoprotectant-treated specimen in liquid nitrogen, has the advantages of technical simplicity as elaborate controlled-cooling equipment is not required. However, they have the

Table 4.22: Survival of cryopreserved embryogenic tissues from two sweet potato genotypes after extended storage periods in liquid nitrogen

Genotype and sucrose treatment	Dehydration (h)	Moisture content (%)	Survival (%)							
			30d storage		60d storage		90d storage		120d storage	
			E	NE	E	NE	E	NE	E	NE
865M(T_1)	0	72-75	0	0	0	0	0	0	0	0
	1.5	37-44	0	0	0	0	0	0	0	0
	3	26-31	33	5	33	5	38	5	24	29
	4	24-29	19	0	10	10	24	10	43	19
	5	24-27	10	5	5	14	19	5	5	5
209M(T_4)	0	74-75	0	0	0	0	0	0	0	0
	1.5	35-44	0	0	0	0	0	0	0	0
	3	25-29	48	14	43	0	62	14	43	10
	4	23-28	19	10	10	0	43	14	52	0
	5	21-27	10	14	5	0	10	0	10	5

n=28

1 Sucrose treatment

Before dehydration / freezing **After freezing/thawing**

T_1: 0.1M(3d); Encapsulation: 0.4M (3d); 0.7M(2d) 0.1M(2d);de-bead;0.1M(28d)

T_4: 0.1M(3d); Encapsulation: 0.4M (3d); 0.7M(2d) 0.4M(2d);de-bead:0.4M(1d):0.1M(28d)

For further experimental details see Table 4.20.

possible disadvantage that there is little scope for the fine adjustment of cooling rates to suit the requirements of particular species or genotypes (Henshaw, 1987). It was evident from the results in the series of experiments in section 4.7 that successful protocols have been developed with the two-step freezing method. Out of five such protocols that were investigated (T_1-T_5), the T_1 sucrose treatment was selected for a rapid freezing procedure. The preliminary experiment was carried out with two genotypes (1023M and 207M). Since the results of that experiment (not described) were unsatisfactory, it was thought that improvements might be made by modifying the encapsulation media. To investigate this possibility, experiments were carried out with genotype 865M in which encapsulated tissue produced by procedures involving different sucrose treatments were subjected to a standard rapid freezing procedure as employed in the previous experiments.

The basic experimental procedure was similar to that used in 4.7.1 (see Fig. 7 and Section 4.7 for details), employing sucrose treatment T_1, except that the embryogenic tissues were encapsulated in media containing different sucrose concentrations (see Table 4.23 for details). They were then subjected to a cooling procedure involving direct transfer to liquid nitrogen.

The data presented in Table 4.23 show that encapsulated medium containing 0.1M sucrose (E_1) produced higher survival rates for frozen tissue than any other encapsulation media, with the tissue subjected to the E_3 and E_4 (0.7 or 1.0M respectively) encapsulation media failing to survive after freezing. With the non-frozen controls, no major differences between the effects of encapsulation media E_1 and E_2 were recorded.

A notable feature of the results in this experiment, compared with those from previous experiments involving a two-step freezing procedure, was the relatively high survival rates for tissues which were not subjected to evaporative dehydration.

Table 4.23:Effect of encapsulation media, evaporative dehydration and rapid freezing on the survival of encapsulated embryogenic tissues of genotype 865M

Dehydration time (h)	Moisture content (%)	Survival (%)							
		E_1				E_2			
		Non-frozen		Rapid freezing		Non-frozen		Rapid freezing	
		E	NE	E	NE	E	NE	E	NE
0		100	0	19	0	62	0	19	0
3		38	10	0	0	33	0	10	0
4		29	0	19	0	14	0	5	0
5		19	0	5	0	5	0	5	0
		E_3				E_4			
0		19	0	0	0	0	0	0	0
3		5	0	0	0	0	0	0	0
4		0	0	0	0	0	0	0	0
5		0	0	0	0	0	0	0	0

n=21

1 Sucrose treatment

Before freezing

0.1M(2d); Encapsulation: 0.4M (3d); 0.7M(2d)

After freezing

0.1M(2d);de-bead;0.1M(28d)

E_1= 0.1M; 3% sodium alginate
E_2= 0.4M; 3% sodium alginate
E_3= 0.7M; 3% sodium alginate
E_4= 1.0M; 3% sodium alginate

2 E = embryogenic, NE = non-embryogenic

3 For further experimental details see Table 4.20

4.13: THE EFFECT OF SUCROSE TREATMENTS, EVAPORATIVE DEHYDRATION AND RAPID FREEZING ON THE SURVIVAL OF ENCAPSULATED EMBRYOGENIC TISSUES FROM SIX SWEET POTATO GENOTYPES

It is evident from the results in the series of experiments in section 4.12 that some success had been achieved in the development of a rapid-freezing cryopreservation procedure that could be used with embryogenic tissue from at least two genotypes. In view of the practical convenience of such a simplified technique a further experiment involving rapid freezing was carried out with six genotypes.

The basic experimental procedure was similar to that used in 4.7.1-4.7.8 (see Fig.7 and section 4.7 for details), employing sucrose treatments T1, T3 and T4 and followed encapsulation procedure E1, followed by the rapid-freezing procedure (see Table 4.24). The results for the individual genotypes are indicated below:

4.13.1: GENOTYPE 865M

The data presented in Table 4.24 show that there were significant differences ($p<0.001$) between the effects of the sucrose treatments, with T1 producing the highest overall survival rates following freezing. None of the surviving tissue following the treatments showed a non-embryogenic response. There were also significant differences between the effects of the dehydration times ($P<0.001$), with 4 h (22-31% moisture contents) producing the highest survival rates.

4.13.2: GENOTYPE 1023M

The data presented in Table 4.25 indicate that there were significant differences ($p<0.001$) between the effects of the sucrose treatments, with T3 producing the highest overall survival rates. In most of the treatments, some of the surviving tissue

Table 4.24: Effect of sucrose treatments, evaporation dehydration and rapid freezing on the survival of encapsulated embryogenic tissues in genotype 865M

Sucrose treatment	Dehydration (h)	Moisture content(%)	Survival (%)	
			Embryogenic	Non-embryogenic
T_1	0	74	29	0
	3	39	5	0
	4	31	19	0
	5	23	29	0
T_3	0	66	5	0
	3	32	5	0
	4	23	14	0
	5	15	5	0
T_4	0	65	19	0
	3	33	0	0
	4	22	19	0
	5	16	5	0

n=21

1 Sucrose treatments:

	Before freezing	**After freezing**
T_1:	0.1M(3d); Encapsulation: 0.4M (3d); 0.7M(2d)	0.1M(2d); de-bead; 0.1M(28d)
T_3:	0.1M(3d); Encapsulation: 0.4M (3d); 0.7M(2d)	0.7M(1d):0.4M(1d):de-bead:0.4M(2d):0.1M(28d)
T_4:	0.1M(3d); Encapsulation: 0.4M (3d); 0.7M(2d)	0.4M(2d); de-bead: 0.4M(1d); 0.1M(28d)

2 Freezing procedure: direct immersion in LN_2 for 1h.
3 Basal Medium: MS+ 5µM 2,4-D
4 Replication: 3 Petri dishes x 7 embryogenic aggregates (n=21)
5 Incubation Conditions: 25°C/16h photoperiod (70µMm^{-2}s^{-1} PAR). Immediately after thawing, all cultures were incubated in the dark at 25°C for 1d.

produced non-embryogenic tissue. There were no significant differences between the effects of the different dehydration times, although the 0 and 5 h dehydration time (11-16% moisture contents) produced the highest survival rates.

4.13.3: GENOTYPE 30MT

The data presented in Table 4.26 show that there were significant differences ($P<0.001$) between the effects of the sucrose treatments, with T3 producing the highest embryogenic survival rates. There were also significant differences between the effects of the dehydration times ($P<0.001$), with 5 h (16-19% moisture contents) overall producing the highest embryogenic survival rates.

4.13.4: GENOTYPE 209M

The data presented in Table 4.27 show that there were no significant differences between the effects of the sucrose treatments. However, there were significant differences ($P<0.001$) between the effects of the dehydration times, with 4 h (15-23% moisture contents) producing the highest overall, embryogenic survival rates.

4.13.5: GENOTYPE 207M

The data presented in Table 4.28 show that there were no significant differences between the effects of the different sucrose treatments on survival. There were also no significant differences between the dehydration times, although the highest overall, embryogenic survival rates was obtained with the 4h treatment (20-28% moisture content). As in previous experiment with the genotype, a relatively high proportion of surviving tissues showed a non-embryogenic response.

Table 4.25: Effect of sucrose treatments, evaporation dehydration and rapid freezing on the survival of encapsulated embryogenic tissues in genotype 1023M

Sucrose treatment	Dehydration time (h)	Moisture content(%)	Survival (%)	
			Embryogenic	Non-embryogenic
T_1	0	71	19	19
	3	19	0	0
	4	14	5	9
	5	11	28	20
T_3	0	65	57	17
	3	26	19	5
	4	22	29	5
	5	16	67	10
T_4	0	65	67	0
	3	29	43	0
	4	24	57	14
	5	16	38	14

n=21

For further experimental details see Table 4.24.

Table 4.26: Effect of sucrose treatments, evaporation dehydration and rapid freezing on the survival of encapsulated embryogenic tissues in genotype 30MT

Sucrose treatment	Dehydration time (h)	Moisture content(%)	Survival (%)	
			Embryogenic	Non-embryogenic
T_1	0	69	5	0
	3	23	0	0
	4	19	5	0
	5	16	19	0
T_3	0	73	7	0
	3	21	0	0
	4	21	0	0
	5	19	7	0
T_4	0	74	4	0
	3	20	18	0
	4	18	10	0
	5	16	14	4

n=21

For further experimental details see Table 4.24.

Table 4.27: Effect of sucrose treatments, evaporation dehydration and rapid freezing on the survival of encapsulated embryogenic tissues in genotype 209M

Sucrose treatment	Dehydration time (h)	Moisture content(%)	Survival (%)	
			Embryogenic	Non-embryogenic
T_1	0	71	29	9
	3	26	38	0
	4	23	14	5
	5	21	5	0
T_3	0	73	18	0
	3	18	0	0
	4	15	43	4
	5	14	50	4
T_4	0	73	25	4
	3	17	0	0
	4	15	64	0
	5	14	50	4

n=21

For further experimental details see Table 4.24.

Table 4.28: Effect of sucrose treatments, evaporation dehydration and rapid freezing on the survival of encapsulated embryogenic tissues in genotype 207M

Sucrose treatment	Dehydration time (h)	Moisture content(%)	Survival (%)	
			Embryogenic	Non-embryogenic
T_1	0	75	0	0
	3	29	0	0
	4	28	14	5
	5	27	28	14
T_3	0	73	0	0
	3	24	0	14
	4	21	19	29
	5	20	5	14
T_4	0	74	0	0
	3	23	5	10
	4	20	19	28
	5	20	5	10

n=21

For further experimental details see Table 4.24.

4.13.6: GENOTYPE 132M

The data presented in Table 4.29 show that there were no significant differences between the effects of different sucrose treatments on survival rate. There were, however, significant differences ($p<0.05$) between the effects of the dehydration times, with 4h (23-25% moisture content)producing the highest embryogenic survival rates.

4.13.7: GENERAL CONCLUSIONS FROM EXPERIMENTS 4.13.1- 4.13.6

1. There was some survival of embryogenic tissue following the rapid-freezing procedure with all of the genotypes ranging between means of 51% (genotype 1023M) and 12% (genotype 30MT).
2. As in the previous experiment (4.12.1), some relatively high survival rates were obtained with tissue that had not been subjected to evaporative dehydration.

4.14 THE EFFECT OF CRYOPRESERVATION PROTOCOLS ON THE SURVIVAL OF NON-ENCAPSULATED SWEET POTATO EMBRYOGENIC TISSUE

The previous experiment had been designed with the intention of studying the effectiveness of cryopreservation protocols involving combination of sucrose, encapsulation and evaporative dehydration treatments with the sweet potato embryogenic tissue.

Although some success was obtained with this approach there remained the question of whether the process could be simplified for practical purposes by the omission of the encapsulation stage. Two separate series of experiments were therefore, carried out in which non-encapsulated embryogenic tissues were subjected, respectively, to two-step and rapid-freezing procedures:

Table 4.29: Effect of sucrose treatments, evaporation dehydration and rapid freezing on the survival of encapsulated embryogenic tissues in genotype 132M

Sucrose treatment	Dehydration time (h)	Moisture content(%)	Survival (%)	
			Embryogenic	Non-embryogenic
T_1	0	73	5	0
	3	30	0	0
	4	25	5	0
	5	22	5	0
T_3	0	73	24	9
	3	25	25	0
	4	23	32	14
	5	22	25	11
T_4	0	74	33	0
	3	26	4	0
	4	23	29	7
	5	23	11	4

n=21

For further experimental details see Table 4.24.

4.14.1 THE EFFECTS OF SUCROSE TREATMENTS, EVAPORATIVE DEHYDRATION AND TWO-STEP FREEZING ON THE SURVIVAL OF NON-ENCAPSULATED EMBRYOGENIC TISSUES FROM FOUR SWEET POTATO GENOTYPES

The basic experimental procedure was similar to that employed in 4.7.1-4.6.7 (see Fig. 7 and 4.7 for details), omitting the encapsulation procedure and making the following changes:

a. After the pre-freezing treatments, the embryogenic aggregates were transferred to a sterilized filter paper carrier (2.0 x 0.5cm) and dehydrated in petri dishes containing silica-gel (2.0g per dish) for various times. The filter paper carrier was placed in a 2ml cryovial before a two-step freezing process
b. Three sucrose treatments were selected for the investigation from among those used in experiments 4.7.1-4.7.8, using the same codes.

4.14.1.1 Genotype 865M

The data presented in Table 4.30 show that there were significant differences ($p<0.001$) between the effects of the sucrose treatments, with T_2 producing the highest embryogenic survival rates. There were also significant differences between the effects of the dehydration times ($p<0.001$), with 1.5h producing the highest embryogenic survival rates. With non-frozen controls, there were significant differences ($p<0.05$) between the effects of the sucrose treatments, with T_2 producing the highest embryogenic survival rates. There were also significant differences between the effects of the dehydration times ($p<0.05$), with 1.5h (18-25% moisture content) producing the highest embryogenic survival rates.

Table 4.30: Effect of sucrose treatments, evaporation dehydration and two-step freezing on the survival of non-encapsulated embryogenic tissues in genotype 865M

Sucrose treatment/ dehydration time (h)	Moisture content(%)	Survival (%)			
		Non-frozen		Two-step freezing	
		E	NE	E	NE
T_1 0	69-70	100	0	0	0
1.5	18-20	76	0	24	5
3	16-18	0	38	0	0
4	14-15	0	0	0	0
5	14	0	0	0	0
T_2 0	69-71	100	0	0	0
1.5	19-22	95	0	81	5
3	18-19	14	0	29	4
4	17-18	5	0	5	24
5	17	0	0	0	0
T_4 0	70-71	100	0	0	0
1.5	24-25	81	9	38	0
3	16-18	14	0	14	0
4	15-16	5	0	14	0
5	15	0	0	0	0

n=21

1 Sucrose treatments:

	Before dehydration / freezing	**After freezing / thawing**
T_1:	0.1M (3d); 0.4M (3d); 0.7M (2d)	0.1M (2d);0.1M (28d)
T_2:	0.1M (3d); 0.4M (3d); 0.7M (2d); 1.0M (2d)	0.1M (2d) ; 0.1M (28d)
T_4:	0.1M (3d); 0.4M (3d); 0.7M (2d)	0.4M (2d); 0.4M(1d); 0.1M(28d)

2 E = embryogenic, NE = non-embryogenic

3 Freezing procedure: ambient temperature to 0°C at 10°C min^{-1}; 0°C to –40°C at 0.5°C min^{-1}, followed by transfer to LN_2 for 1h.

4 Basal Medium: MS+ 5µM 2,4-D

5 Replication: 3 petri dishes x 7 embryogenic aggregates (n=21)

6 Incubation Conditions: 25°C/16h photoperiod (70µM$m^{-2}s^{-1}$ PAR). Immediately after thawing, all cultures were incubated in the dark at 25°C for 1d.

4.14.1.2 Genotype 1023M

The data presented in Table 4.31 show that there were significant differences ($p<0.001$) between the effects of the sucrose treatments, with T_1 producing the highest embryogenic survival rates. There were also significant differences between the effects of the dehydration times ($p<0.001$), with 1.5h (18-21% moisture content) producing the highest embryogenic survival rates.

With non-frozen controls, there were significant differences ($p<0.001$) between the effects of the sucrose treatments, with T_1 producing the highest embryogenic survival rates. There were also significant differences between the effects of the dehydration times ($p<0.001$), with 1.5h producing the highest embryogenic survival rates.

4.14.1.3 Genotype 30MT

The data presented in Table 4.32 show that there were significant differences ($p<0.05$) between the effects of the sucrose treatments, with T_2 producing the highest embryogenic survival rates. There were also significant differences between the effects of the dehydration times ($p<0.01$), with 1.5h (15-21% moisture content) producing the highest embryogenic survival rates.

With non-frozen controls, there were significant differences ($p<0.01$) between the effects of the sucrose treatments, with T_2 producing the highest embryogenic survival rates. There were also significant differences between the effects of the dehydration times, with 1.5h producing the highest embryogenic survival rates.

4.14.1.4 Genotype 209M

The data that is presented in Table 4.33 would strongly indicate that there were significant differences ($p<0.01$) between the effects of the sucrose treatments, with the T_3 sucrose treatment producing the highest embryogenic survival rates. There were

Table 4.31: Effect of sucrose treatments, evaporation dehydration and two-step freezing on the survival of non-encapsulated embryogenic tissues in genotype 1023M

Sucrose treatment/ dehydration time (h)	Moisture content(%)	Survival (%)			
		Non-frozen		Two-step freezing	
		E	NE	E	NE
T_1 0	65-66	100	0	0	0
1.5	18-20	86	14	81	5
3	17-18	19	29	86	5
4	16-17	0	52	10	0
5	16	0	0	0	0
T_2 0	62-65	100	0	0	0
1.5	20-21	10	0	0	0
3	17-18	0	0	0	0
4	17	0	0	0	0
5	10	0	0	0	0
T_4 0	69-70	100	0	0	0
1.5	18-19	38	14	10	0
3	17-18	0	0	0	0
4	14-16	0	0	0	0
5	9	0	0	0	0

n=21

For experimental details see Table 4.30

Table 4.32: Effect of sucrose treatments, evaporation dehydration and two-step freezing on the survival of non-encapsulated embryogenic tissues in genotype 30MT

Sucrose treatment/ dehydration time (h)	Moisture content(%)	Survival (%)			
		Non-frozen		Two-step freezing	
		E	NE	E	NE
T_1 0	73-74	95	5	0	0
1.5	16-17	10	0	0	0
3	11-15	0	0	0	0
4	10-11	0	0	0	0
5	8	0	0	0	0
T_2 0	66-68	76	0	0	0
1.5	20-21	57	0	24	0
3	17-18	43	9	0	0
4	15-16	5	0	0	0
5	10	5	0	0	0
T_4 0	71-72	76	5	0	0
1.5	15-16	67	0	0	0
3	14-15	0	0	14.0	0
4	12-13	0	0	0	0
5	12	0	0	0	0

n=21

For experimental details see Table 4.30

Table 4.33: Effect of sucrose treatments, evaporation dehydration and two-step freezing on the survival of non-encapsulated embryogenic tissues in genotype 209M

Sucrose treatment/ dehydration time (h)	Moisture content(%)	Survival (%)			
		Non-frozen		Two-step freezing	
		E	NE	E	NE
T_1 0	74-76	81	0	0	0
1.5	17-18	57	0	10	0
3	14-16	43	0	0	0
4	13-14	0	0	0	0
5	13	0	0	0	0
T_2 0	62-64	57	43	0	0
1.5	17-19	43	5	5	0
3	16-17	0	0	0	0
4	15-16	0	0	0	0
5	15	0	0	0	0
T_4 0	73-74	100	0	0	0
1.5	28-29	43	5	33	19
3	25-28	24	5	0	5
4	20-24	0	10	10	14
5	17	5	0	0	0

n=21

For experimental details see Table 4.30

also significant differences between the effects of the dehydration times ($p<0.001$), with 1.5h (17-29% moisture contents) producing the highest embryogenic survival rates. With non-frozen controls, there were significant differences ($p<0.05$) between the effects of the sucrose treatments, with T_3 producing the highest embryogenic survival rates. There were also significant differences between the effects of the dehydration times, with 1.5h producing the highest embryogenic survival rates.

4.14.1.5: General conclusions from experiments 4.14.1- 4.14.4

1 There was some survival of embryogenic tissue following the freezing procedure with all of the genotypes, ranging between means of 44% (genotype 1023M) and 6% (genotype 30MT).

2 There was no consistent pattern of successful treatments among the various genotypes, although the T_2 sucrose treatment and 1.5h dehydration time produced some of the best embryogenic survival rates.

4.14.2 THE EFFECT OF SUCROSE TREATMENTS, EVAPORATIVE DEHYDRATION AND RAPID FREEZING ON THE SURVIVAL OF NON-ENCAPSULATED EMBRYOGENIC TISSUES FROM FOUR SWEET POTATO GENOTYPES

The basic experimental procedure was similar to that employed in 4.14.1 (see section 4.14 for details) except that a rapid freezing procedure (see section 4.14.1 for details) was substituted for the two-step freezing procedure.

4.14.2.1 Genotype 865M

The data that is presented in table 4.34 indicates that there were significant differences ($p<0.001$) between the effects of the sucrose treatments, with the T_4 sucrose treatment producing the highest embryogenic survival rates of embryogenic tissue. There were

Table 4.34: Effect of sucrose treatments, evaporation dehydration and rapid freezing on the survival of non-encapsulated embryogenic tissues in genotype 865M

Sucrose treatment	Dehydration (h)	Moisture content (%)	Survival (%)	
			Rapid freezing	
			Embryogenic	Non-embryogenic
T_1	0	69	0	0
	1.5	18	81	0
	3	17	0	0
	4	15	0	0
	5	14	0	0
T_2	0	69	0	0
	1.5	19	76	0
	3	18	10	0
	4	18	0	0
	5	17	0	0
T_4	0	70	5	0
	1.5	25	81	10
	3	16	0	0
	4	15	0	0
	5	15	0	0

n=21

1 Sucrose treatments:

	Before dehydration / freezing	**After freezing / thawing**
T_1:	0.1M (3d); 0.4M (3d); 0.7M (2d)	0.1M (2d);0.1M (28d)
T_2:	0.1M (3d); 0.4M (3d); 0.7M (2d); 1.0M (2d)	0.1M (2d) : 0.1M (28d)
T_4:	0.1M (3d); 0.4M (3d); 0.7M (2d)	0.4M (2d); 0.4M(1d); 0.1M(28d)

2 Freezing procedure: All samples were directly immersed in liquid nitrogen for 1h

For further experimental details see Table 4.30

also significant differences between the effects of the dehydration times ($p<0.001$), with 1.5h (17-21% moisture contents) producing the highest embryogenic survival rates.

4.14.2.2 Genotype 1023M

The data presented in table 4.35 show that there were significant differences ($p<0.001$) between the effects of the sucrose treatments, with T_1 producing the highest embryogenic survival rates. There were also significant differences between the effects of the dehydration times ($p<0.001$), with 1.5h (17-21% moisture) producing the highest embryogenic survival rates.

4.14.2.3 Genotype 30MT

The data presented in table 4.36 show that there were significant differences ($p<0.05$) between the effects of the sucrose treatments, with T_2 producing the highest embryogenic survival rates. There were also significant differences between the effects of the dehydration times ($p<0.1$), with 1.5h producing the highest embryogenic survival rates.

4.14.2.4 Genotype 209M

The data presented in table 4.37 indicate that there were significant differences ($p<0.01$) between the effects of the sucrose treatments, with T_3 producing the highest embryogenic survival rates. There were also significant differences between the effects of the dehydration times ($p<0.001$), with 1.5h (15-21% moisture content) producing the highest embryogenic survival rates.

Table 4.35: Effect of sucrose treatments, evaporation dehydration and rapid freezing on the survival of non-encapsulated embryogenic tissues in genotype 1023M

Sucrose treatment	Dehydration time (h)	Moisture content (%)	Survival (%)	
			Rapid freezing	
			Embryogenic	Non-embryogenic
T_1	0	66	0	0
	1.5	18	57	24
	3	17	33	43
	4	17	0	43
	5	16	5	5
T_2	0	62	0	0
	1.5	21	0	0
	3	17	0	0
	4	17	0	0
	5	9	0	0
T_4	0	69	0	0
	1.5	17	14	0
	3	17	0	0
	4	14	0	0
	5	9	0	0

n=21

For further experimental details see Table 4.34

Table 4.36: Effect of sucrose treatments, evaporation dehydration and rapid freezing on the survival of non-encapsulated embryogenic tissues in genotype 30MT

Sucrose treatment	Dehydration time (h)	Moisture content (%)	Survival (%)	
			Rapid freezing	
			Embryogenic	Non-embryogenic
T_1	0	74	5	0
	1.5	16	0	0
	3	11	0	0
	4	10	0	0
	5	8	0	0
T_2	0	66	0	0
	1.5	21	33	5
	3	17	5	0
	4	15	0	0
	5	10	0	0
T_4	0	72	0	0
	1.5	15	29	0
	3	15	0	0
	4	14	0	0
	5	12	0	0

n=21

For further experimental details see Table 4.34

Table 4.37: Effect of sucrose treatments, evaporation dehydration and rapid freezing on the survival of non-encapsulated embryogenic tissues in genotype 209M

Sucrose treatment	Dehydration time (h)	Moisture content (%)	Survival (%)	
			Rapid freezing	
			Embryogenic	Non-embryogenic
T_1	0	76	0	0
	1.5	17	57	0
	3	14	38	0
	4	14	0	0
	5	14	0	0
T_2	0	62	0	0
	1.5	17	14	5
	3	16	0	0
	4	16	0	0
	5	15	0	0
T_4	0	74	0	0
	1.5	29	48	23
	3	25	5	5
	4	20	38	14
	5	17	10	0

n=21

For further experimental details see Table 4.34

4.14.2.5: General conclusions from experiments 4.14.2.1- 4.14.2.4

1 There was some survival of embryogenic tissues, following the freezing procedure with all of the genotypes, ranging between means of 24% (genotype 865M) and 10% (genotype 30MT).

2 There was no consistent pattern of successful treatments among the various genotypes, although the T_1 sucrose treatment and 1.5h dehydration time produced some of the best embryogenic survival rates.

Fig 4 Procedure for experiment 4.1

Three batches of ten embryogenic aggregates were selected for each experimental interaction (5 genotypes x 11 sucrose treatments). Each batch was subjected to the following procedure:

a. The aggregates were transferred through a series of sucrose treatments as indicated under Table 4.1(T_1-T_{11}). For each treatment, the aggregates were placed in a petridish containing 10ml MS medium + 5μM 2,4-D and the appropriate concentrations of sucrose. The petri dishes were incubated in light (16h photoperiod) at 25°C.

b. Following the sucrose treatment, aggregates were transferred to petridishes containing 10ml MS medium + 5μM 2,4-D and 0.1M sucrose in the light (16h photoperiod) at 25°C.

c. After 28d data for survival and embryogenic competence were recorded.

Fig 5 Procedure for experiment 4.2

Three batches of ten embryogenic aggregates were selected for each experimental interaction (4 genotypes x 13 sucrose treatments). The batches were subjected to the following procedure:

a. The aggregates were placed in a petridish containing 10ml MS medium + 5μM 2,4-D and 0.1M sucrose for 2d in the light (16h photoperiod) at 25°C.

b. Each aggregate was encapsulated in an alginate bead (see Section 2.6.2).

c. The beads were transferred through a series of sucrose treatments as indicated in Table 4.2 (T_1-T_{13}). For each treatment, the aggregates were placed in a petridish containing 10ml MS medium + 5μM 2,4-D and the appropriate concentrations of sucrose

d. The beads were transferred to petridishes containing 10ml MS medium + 5μM 2,4-D and 0.1M sucrose in the light (16h photoperiod) at 25°C.

e. The aggregates were removed from the alginate beads (de-beading) and transferred to petridishes containing MS medium + 5μM 2,4-D and 0.1M sucrose in the light (16h photoperiod) at 25°C.

f. After 28d data for survival and embryogenic competence were recorded.

Fig 6 Procedure for experiment 4.3

Three batches of ten embryogenic aggregates were selected for each experimental interaction (6 genotypes x 7 dehydration times). Each batch of aggregates was subjected to the following procedure:

a The aggregates were transferred to a petridish containing MS medium + 5μM 2,4-D and 0.1M sucrose for 2d in the light (16h photoperiod) at 25°C.

b Each aggregate was encapsulated in an alginate bead (see Section 2.6.2).

c The beads were transferred to petridishes containing MS medium + 5μM 2,4-D and 0.4M sucrose for 3d in the light (16h photoperiod) at 25°C.

d The beads were removed from the petridishes and dehydrated for the appropriate time (0-6h).

e 7 beads were transferred to a petridish containing MS medium + 5μM 2,4-D and 0.1M sucrose for 2d in the light (16h photoperiod) at 25°C; 3 beads were used for determination of moisture content.

f The aggregates were removed from beads.

g The aggregates were transferred to a petridish containing MS medium + 5μM 2,4-D and 0.1M sucrose in the light (16h photoperiod) at 25°C.

h After 28d data for survival and embryogenic competence were recorded.

Fig 7. Procedure for experiment 4.6

Three batches of ten embryogenic aggregates were selected for each experimental interaction (1 genotypes x 3 dehydration times x frozen and unfrozen treatments). Each batch was subjected to the following procedure:

a The aggregates were transferred to a petridish containing MS medium + 5μM 2,4-D and 0.1M sucrose for 3d in the light (16h photoperiod) at 25°C.

b Each aggregate was encapsulated in an alginate bead (see Section 2.6.2).

c The beads were transferred to petridishes containing MS medium + 5μM 2,4-D and 0.4M sucrose for 2d in the light (16h photoperiod) at 25°C and then to MS medium + 5μM 2,4-D and 0.7M sucrose for 2d.

d The beads were removed from the petridishes and dehydrated for the appropriate time (0-6h); 3 beads were used to determine moisture content.

e The remaining 7 beads were subjected to a two-step freezing procedure (except for unfrozen controls): beads transferred to 2ml cryovials and slow cooled to 0°C at 10°C min^{-1} and from 0°C to –40°C at 0.5°C min^{-1}, followed by rapid cooling by direct immersion in LN_2.

f After storage in LN_2 for 1h, the beads were thawed rapidly by transfer of the cryovials to a water bath at 40°C for 2m.

g The beads were transferred to a petridish containing MS medium + 5μM 2,4-D and 0.1M sucrose for 1d in the dark, followed by 2d in the light (16h photoperiod) at 25°C.

h The aggregates were removed from the beads.

i The aggregates were transferred to a petridish containing MS medium + 5μM 2,4-D and 0.1M sucrose in the light (16h photoperiod) at 25°C.

j After 28d data for survival and embryogenic competence were recorded.

4.15 DISCUSSION

Since previous work (Blakesley, pers. com.; Towill and Jarret, 1992; de Goes, 1993) had clearly demonstrated the recalcitrance of sweet potato meristems subjected to some of the standard cryopreservation procedures, a decision was taken for practical reasons in the present programme to carry out the initial investigations with the more readily available embryogenic tissues before making any further attempt with the meristems. The first task therefore, was to establish the response of the embryogenic tissues to the basic cryoprotection procedures (sucrose treatments, evaporative dehydration and encapsulation) that were to be used in conjunction with the freezing process. These procedures, which in principle were similar to those used in sweet potato by Blakesley et al, (1995), followed a number of relatively recent studies by several other workers (Fabre and Dereuddre, 1990; Nino and Sakai, 1992; Plessis et al, 1993; Dumet et al, 1993: Gonzalez- Arnao et al, 1996). In these studies sucrose was used as a cryoprotectant in combination with gel-encapsulation and evaporative dehydration procedures for the survival of water from the tissues.

In a preliminary experiment, aimed at establishing the tolerance of embryogenic tissues to different sucrose treatments that might be employed for cryoprotection procedures (see Section 4.1), the eight genotypes that were tested, responded differently to different treatments (0.1M to 1.0M for 1-5d). Overall, the highest sucrose concentration that did not cause excessive damage to embryogenic tissues was 0.4M for 1-5d (80-100% survival of embryogenic tissues). The most effective combined sucrose and encapsulation treatment (see section 4.2) with three out of four genotypes, also involved the use of 0.4M sucrose for periods of exposure up to 5d (67-87% survival of embryogenic tissues). The first sign of damage following exposure to a higher sucrose concentration (0.7M)

was a loss of embryogenic competence, resulting in the production of non-embryogenic callus tissue from surviving cells. With exposure to an even higher sucrose concentration (1.0M), there was a complete loss of viability. The reasons for the loss of viability are not understood, bur they might include damage to plasmodesmetal connections between cells and/or to the cytoskeleton within the cells.

It was also noted in this experiment that encapsulated embryogenic tissues exposed to a low concentration of sucrose (0.1M) for up to 20d following re-hydration showed a loss of embryogenic competence. Following this observation, it was decided in future experiments to remove the tissues from the gel-bead at an early stage (2d after thawing) before further incubation on the recovery medium.

According to the same criteria as used previously for judging the suitability of treatments, encapsulated embryogenic tissues incubated with 0.4M sucrose for 3d before being exposed to periods of evaporative dehydration of up to 6h, gave the highest survival rates with 3h dehydration (moisture contents 24-41%). The tissues did not show excessive levels of damage until the dehydration period exceeded 5h (moisture contents 12-16%). With the longer periods of dehydration, and particularly when the content was less than 16%, there was in general a relatively high loss of embryogenic competence. Below 14% moisture content, there was a complete loss of viability (see section 4.3).

Following the results obtained in section 4.3, it was decided that the use of a more extended sucrose pre-treatment period involving 3d at 0.4M, as in the previous experiment, followed a further 2d at 0.7M prior to evaporative dehydration for the periods of up to 6h should be investigated with six sweet potato genotypes (see section 4.4). In this case, dehydration periods of up to 5h, producing moisture contents in the

range of 14-16% gave the higher survival rates (10-81%) of embryogenic tissues compared with survival rates of (14-29%) of embryogenic tissue in previous experiments. With longer periods of dehydration, the tissue again lost embryogenic competence and eventually when moisture content fell below 14%, the survival rate was reduced to 3-48% (see section 4.4).

On the basis of results obtained in section 4.4, a protocol involving a same gradual increase in sucrose concentration, over a period of 8d, prior to evaporative dehydration and a gradual decrease in sucrose levels after re-hydration was investigated with one sweet potato genotype (1023M). In between the sucrose treatments, the encapsulated embryogenic tissues underwent evaporative dehydration for periods of up to 6h (final moisture contents 15%) (see section 4.5). In this case, a higher survival of embryogenic tissues (100%) was obtained with up to 5h dehydration (moisture contents 18%) than with equivalent levels of dehydration in previous experiment (10-81%) survival (see section 4.4-4.5).

In previously published work, sucrose has been widely reported to be effective in inducing tolerance to dehydration, but the mechanism of sucrose action in this report is not fully understood. Pre-culture on high sucrose media results in the accumulation of sugar in the alginate bead and in the tissue. It has been suggested (Crowe et al, 1984; 1990) that the accumulation of sugar in the tissue maintains membrane stability in the dehydrated state by replacing water molecules at the charged exterior surface of membranes. In many cases, the encapsulation-dehydration protocol simply requires a short pre-culture period with an elevated level of sucrose. For example Gonzalez-Arnao et al, (1996) pre-cultured sugarcane apices on 0.75M sucrose for just 24h prior to

dehydration, because longer pre-culture periods inhibited survival after re-hydration. In contrast, it was found that a stepwise increase in sucrose was necessary for optimum protection of encapsulated embryogenic tissue of sweet potato genotype TIB10 (Blakesley et al, 1995). In the present study, the stepwise changes in sucrose concentration both before and after dehydration for periods of up to 6h were necessary for optimum protection of dehydrated encapsulated embryogenic tissue of sweet potato genotype 1023M. This compared with a previous experiment (see section 4.3) in which sucrose concentrations were not changed progressively or were only changed in this manner before dehydration.

In the next experiment, the successful protocol involving sucrose pre-treatment, encapsulation and evaporative dehydration developed with one genotype (1023M, see section 4.4-4.5) was investigated in combination with a two-step freezing process and the same genotype (see section 4.6). Following this, five different sucrose treatments, together with the two-step freezing process, were employed in turn with eight sweet potato genotypes in a series of separate experiments (see section 4.7). This led to a further experiment in which a slightly modified version of one of the more successful protocols was employed with six sweet potato genotypes in a single experiment (see section 4.8). Finally, to test further the reproducibility of the results, protocols which produced some of the best and the poorest survival rates in the previous experiments were applied to tissues from four sweet potato genotypes in a single experiment (see section 4.9).

Overall, there was a genotype effect on survival of two-step freezing, and also variation in the responses of the genotypes to the pre-culture sucrose treatments. Five of the eight

sweet potato genotypes gave survival rates of 67% and a further two in excess of 33%, all after evaporative dehydration (Plate 5). The most effective sucrose treatment varied with the genotype, and no individual sucrose treatment was significantly superior to any other. However, with the exception of 865M and 132M, sucrose treatment 3, involving stepwise changes in sucrose concentration both before and after the freezing and thawing sequence, produced good survival rates (mean=22%) (see section 4.7). It is not possible to identify an optimum length of evaporative dehydration which would give maximum survival for any genotype as there was no significant interaction between genotype and dehydration time. No survival of freezing was recorded without evaporative dehydration (data not shown) except with genotype 865M, for which 9% survival of embryogenic tissues was recorded with sucrose treatment 3.

In other cryopreservation studies, combinations of treatments involving high sucrose levels and evaporative dehydration in combination with two-step freezing e.g. coconut somatic embryos (Assy-Bath and Engelmann, 1992), coffee and carrot somatic embryos (Tessereau et al, 1994), walnut somatic embryos (Boucaud et al, 1994). More recently, Blakesley, et al (1995) reported that embryogenic tissues of two sweet potato genotypes TIB10 and Nem survived the two-step freezing process with rates in the range of 26-74%. The best survival was obtained from tissue exposed to a gradual increase in sucrose concentration, prior to freezing, without the gradual decrease in sucrose concentration following, which was employed in the present study. In this three out of eight genotypes showed survival rates in excess of 67%. Dehydration improved the survival of embryogenic tissue, with a 4h period producing the best results (Blakesley et al, loc.cit.). In the present study, all of the genotypes survived the two-step freezing process all after

3-5 h dehydration periods, and five of the eight genotypes showed survival rates in excess of 67%, with the other three showing rates in the range 14-43%.(Plate 5).

The type of genotypic variation in the responses to the cryopreservation protocols that was observed in the first series of experiments was not unexpected, since there has been common experience with many plant species as indicated very clearly in the results obtained by Schafer-Menuhr et al (1997) with 125 potato genotypes, and it is important for practical purposes that protocols consistently capable of producing acceptable results with a wide a range of genotypes should be identified. The following experiments (see section 4.9) in which a more limited number of sucrose/ dehydration protocols, selected on the basis of their performance in the previous series of experiments (see section 4.7), were applied simultaneously to several genotypes again demonstrated the extent of variation experiments, however, showed inconsistencies in the patterns of responses. For example, the responses to the various sucrose treatments of genotypes 30MT, 1023M and T1B10 (see section 4.9) were found to be broadly similar to those obtained in previous experiments (see section 4.7), but actual survival rates with similar treatments varied between the experiments. Such inconsistencies in the survival rates of embryogenic tissues in the different experiments might have been the results of factors such as differences in the culture ages or in the sizes and compositions of the embryogenic aggregates that were used,. The latter, in particular is very difficult to control because of the heterogeneous mature of all callus cultures, especially those containing embryogenic tissues.

Because, it was thought that variations in culture age might have been affecting the responses of tissues to the various cryopreservation protocols, an experiment (see section

4.10) was designed specifically to test this possibility. Embryogenic tissues form two genotypes taken at different times following subculture were therefore subjected to a standard protocol based on one of those employed in the previous experiments. The results demonstrated that there were significant differences in the responses of tissues of different ages but they were relatively small. This, combined with the fact that the two genotypes responded differently, indicated that the very considerable effort that would be required to select tissues of a particular age for further experiments would not be justified. Accordingly, the tissue of different ages in range 14 to 28d following subculture were routinely used in all of the subsequent experiments on a randomized basis. It can be concluded that other factors must have been responsible for the observe inconsistencies between the results obtained in the different experiments.

It is generally believed that tissues stored at the temperature of liquid nitrogen are not adversely effected by prolonged storage periods, although this is based more on theoretical considerations than on wide practical experience. The effects of storage periods of up to 120d in liquid nitrogen on the viability of tissue were therefore investigated with two sweet potato genotypes (see section 4.11). Two successful protocols developed in the previous studies, and involving a two-step freezing process, were used to prepare the tissues for storage. The results demonstrated that no significant effects of the extended storage periods were recorded on the viability and embryogenic competence of frozen tissue in either of the genotypes.

No previous work has been reported concerning the effect of extended storage periods at the temperature of liquid nitrogen on the survival of sweet potato tissues. Bazaz (1976) however, demonstrated with cryopreserved globular embryos from carrot cell suspension

cultures that plants of normal appearance, producing healthy roots, were grown from cultures stored for up to eight months, although the survival of embryos decreased with advances in their stage of development. More recently, in a very extensive investigation, Schafer-Menuhr et al (1997) reported that the rates of survival (means=80%) and plant regeneration (mean=38%) from cryopreseved meristems of 125 potato genotypes stored for up to three years were unchanged compared with the values obtained immediately after freezing, and there was no evidence of enhanced somaclonal variations among the regenerated plants.

It is evident that more extensive long-term cryostorage experiments, along the lines of those reported by Schafer-Menuhr et al (loc. cit.), are required with a wider range of species. It appears, however, that the assumption about the long-term survival of cryopreserved tissues might be well founded. The experiment described here with sweet potato, although limited in cope, does not conflict with that conclusion. Certainly, the available evidence supports the view that cryostorage techniques could provide a valuable alternative strategy for the long-term storage of sweet potato germplasm, assuming that efficient, relatively simple protocols can be developed.

In comparison with two-step freezing procedures, rapid-freezing procedures have the considerable advantage of technical simplicity, thus avoiding the need for expensive controlled-cooling equipment. It was therefore decided that one of the cryoprotection protocols used successfully in combination with the two-step freezing procedure would be investigated in combination with a rapid-cooling method. The results of a preliminary study (not shown) were not satisfactory and it was thought that improvements might be made by modifying the encapsulation medium, according to the procedure used by other

workers (Hatanaka et al, 1994) in which this medium contained higher concentration of sucrose. Alginate beads were therefore prepared from media containing different sucrose concentrations (0.1-11.0M) and tested with one sweet potato genotype, using one of the cryoprotection sequences successfully employed in the two-step freezing (see section 4.12). Using beads in the same manner, a further experiment was carried out with six sweet potato genotypes, and three of the cryoprotection protocols employed previously in the two-step freezing investigations (see section 4.13).

The results of the first study demonstrated that the best survival (19%) of embryogenic tissue following rapid freezing was obtained with beads prepared with 0.1M sucrose. There were no significant differences between the results obtained with the beads prepared with 0.1M and 0.4M sucrose in terms of the survival of embryogenic tissue. However, the beads prepared with the highest sucrose concentrations (0.7M-1.0M) showed poor survival rates, even in the unfrozen controls. In the subsequent rapid-freezing experiments, therefore, the beads were prepared according to the procedure used previously in the two-step freezing experiments.

The results of the second rapid freezing study (see section 4.13) indicated that encapsulated embryogenic tissues from all of the six tested genotypes survived rapid-freezing, with rates ranging from 16-67%. Two of the six genotypes showed survival rates in excess of 64%. In general, the evaporative dehydration sequence did not improve the survival of embryogenic tissues, compared with the non-dehydrated controls. In addition, survival rates declined with 3-4h dehydration only to recover with the longer dehydration periods (5h)-but not beyond the 0h rates.

In previously published work concerning sweet potato, Blakesley et al (1995) reported that up to 28% of encapsulated embryogenic tissues of genotype T!B10 survived rapid-freezing without dehydration. This protocol was based on a stepwise increase in sucrose concentrations before freezing, and direct transfer to 0.1M for re-hydration after thawing. In comparison, in the present study, two of the six genotypes gave considerably higher survival rates when a similar protocol was followed except that stepwise changes in sucrose concentrations were applied both before and after the freezing/ thawing sequence. This perhaps emphasises the need to pay more attention to recovery conditions during the period that the tissues are stressed following thawing.

In the experiments reported here, therefore, some success was achieved in applying the rapid-freezing technique to encapsulated embryogenic tissues with six sweet potato genotypes. Again, as found previously in the experiments involving the two-step freezing process, the genotypes differed in their responses to the various sucrose treatments. Also, there remained the additional question of whether the procedure could be simplified even further by omitting the encapsulation technique. Before finishing the investigation with the embryogenic tissue therefore, two further experiments (see section 4.14) were carried out comparing the two-step and rapid freezing procedures applied to non-encapsulated embryogenic tissues. In each of the experiments, three sucrose treatments used successfully in the experiments were employed with four sweet potato genotypes (see section 4.14.1 and 4.14.2).

The results of the first experiment demonstrated that non-encapsulated embryogenic tissues from all of the genotypes survived the two-step freezing process with rates ranging from 24-86%, with two of the genotypes showing particularly high survival rates

(81% and 86%). Explants dehydrated for 1.5h (moisture contents 15-29%) with silica gel showed the highest survival rates. In the earlier experiments, encapsulated embryogenic tissues did not survive the two-step freezing process after 1.5h dehydration (results not shown) and 3-5h dehydration (moisture contents 11-34%) was required to produce optimal survival. These differences are probably the result of the more rapid loss of water from the non-encapsulated tissues during the evaporation treatments carried out under different conditions, as indicated by the similarities in the moisture contents after the respective dehydration periods.

The results of the second study demonstrated that some of the non-encapsulated embryogenic tissues from all of the genotypes survived the rapid freezing process, with rates ranging from 33-81%. Three of the four genotypes showed high survival rates between 57% and 81%. The trends in the responses to dehydration of non-encapsulated tissues subjected to rapid freezing and two-step freezing processes in the two experiments were similar, with the 1.5hrs dehydration period producing the highest survival rates. Without dehydration, however non-encapsulated embryogenic tissues did not survive rapid freezing, in contrast to the results obtained previously with encapsulated embryogenic tissues (see section 4.13), which produced the highest survival rates under those conditions.

In recently published work, Blakesley et al (1996) have reported that with the sweet potato genotype T1B10, up to 50% of non-encapsulated embryogenic tissues survived a rapid-freezing process, after pre-culture on 0.4M or 0.7M sucrose. They also stated that dehydration with silica-gel to moisture contents in the range 18-41% improved the survival after freezing. Later Blakesley et a (1997) reported that non-encapsulated

embryogenic tissues from six sweet potato genotypes showed survival rates in the range of 10-83%, following rapid freezing without dehydration. In the present study, three of the four genotypes showed survival rates in the range 57-86% when rather similar protocols were used except that the post-thaw sucrose treatments involved some gradual decrease in concentration over a period of 3d. Also, in contrast to the results obtained by Blakesley et al (loc. cit.), the non-encapsulated embryogenic tissues did not survive the rapid-freezing process if dehydration was not employed. It is difficult to explain these differences in the responses of the non-dehydrated encapsulated tissues in the two sets of experiments. However, there were differences in the experimental procedures, particularly with regard to the sequences of sucrose treatments during the pre-freezing and post-thawing stages. Clearly, given the potential value of the simplified rapid freezing techniques these aspects of the protocols merit a more extensive investigation. Comparing the two cryopreservation protocols involving, respectively, the application of the rapid and two-step freezing process and the non-encapsulated embryogenic tissues (see section 4.14), the rapid freezing was found to be somewhat more effective in terms of the higher survival rates that were obtained. As it could not be claimed, however, that either of these processes had been fully optimized with the various genotypes, it would be premature to assume that the rapid freezing process would consistently produce the better results.

When the rapid and two-step freezing processes were used with the encapsulated embryogenic tissue, the most effective sucrose treatment involving stepwise increase in the sucrose concentrations up to 0.7M over 8d prior to freezing was generally found to be the most effective together with the 3-5h dehydration periods (moisture contents 11-34%). With the rapid freezing process, the highest survival rates were obtained with

sucrose treatments involving stepwise increase in the sucrose concentrations (0.1 to 0.7M over 8d) prior to dehydration and freezing, and gradual decreases in sucrose concentrations following thawing. Survival rates of tissues subjected to evaporative dehydration prior to freezing did not show any improvement. Compared with those of non-encapsulated tissues, the two-step freezing process produced the highest survival rates, compared with rapid freezing.

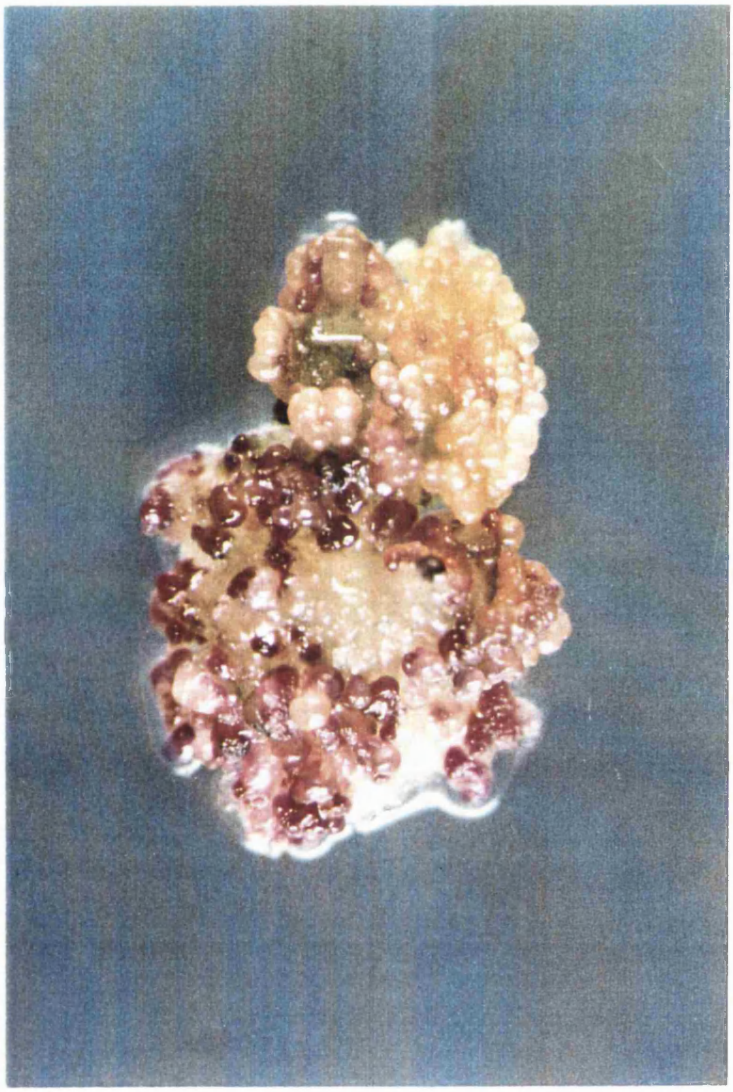
Overall, therefore, as far as the sweet potato embryogenic tissue is concerned, the highest survival rates were obtained with cryopreservation protocols involving both rapid and two-step freezing processes. As far as encapsulated embryogenic tissues are concerned, the two-step freezing process seems to be reliable in terms of high survival rates with a wider range of genotypes. However, since the rapid-freezing procedure in combination with non-encapsulation have a considerable advantage in terms of technical simplicity, it would be important to conduct further work aimed at improving their reliability with a wider range of genotypes.

Plate 5: Survival of embryogenic tissues after two-step freezing process

(x20)

Encapsulated embryogenic tissues pre-cultured on a series of MS media supplemented with 5μM 2,4-D and stepwise increases in sucrose concentration (up to 0.7M) for 8d; dehydrated for 4h frozen by two-step cooling process; transferred to MS medium supplemented with 0.1M sucrose for 24h in dark followed by 24h light at 25°C; harvested from beads; transferred to MS medium supplemented with 0.1M sucrose for four weeks.

5.



5.0 CRYOPRESERVATION OF SHOOT BUDS

Cryopreservation of sweet potato shoot buds may be a useful technique for the long-term storage of vegetatively propagated plant germplasm (Towill et al, 1988). A single report of the successful cryopreservation of sweet potato shoot meristems (Towill and Jarret, 1992) and work at Bath (de Goes, 1993) had clearly indicated difficulties in developing a technique with meristems. In view of the difficulties reported by the other workers, it was decided that the techniques developed with the apparently more responsive embryogenic tissues (see Chapter 4.0) would be used as a basis for studies with shoot tissue. Investigations into cryoprotectants, pre and post-freezing media, sucrose, encapsulation-dehydration, rapid and two-step freezing were therefore undertaken.

5.1: THE EFFECT OF PLANT GROWTH REGULATORS ON PLANT REGENERATION FROM SHOOT BUDS OF TWO SWEET POTATO GENOTYPES

Since it is essential that shoot buds recovering from a cryopreservation protocol should be cultured under optical conditions, this experiment was designed to investigate the effects of different plant growth regulators on the growth and development of shoot buds in the two genotypes.

For each combination from six PGR's (T_1-T_6) and two genotypes 865M and 30MT), ten shoot buds were placed in each of the three replicate petri dishes containing MS medium with 0.06M sucrose and maintained at 25°C in the light (16h photoperiod) for each treatment (see Table 5.1 for further details) for 28d. Plant development was monitored every 7d and final data were recorded after 28d.

The data presented in Table 5.1 indicate that there were significant differences between the effects of the different PGR treatments ($p<0.05$). The combination of 1μM GA_3 +

0.02μM NAA (T_7) produced a higher proportion of plantlets without callus, with each genotype, than any other plant growth regulator treatment. There were no significant differences between the responses of the genotypes, although the genotype 30MT produced more plantlets than 865M. This combination of plant growth regulator was therefore selected for routine use in further experiments.

5.2: THE EFFECT OF CRYOPROTECTION ON THE GROWTH OF SHOOT BUDS FROM TWO SWEET POTATO GENOTYPES

For each combination of the sucrose/glycerol treatments (T_1-T_7) in Table 5.2 and the two genotypes (865M and 30MT), ten shoot buds were placed in each of three replicate Petri dishes containing MS medium supplemented with 1μM GA_3 + 0.02μM NAA and maintained at 25°C in the light (16h photoperiod) (see Table 5.2 for further details). After these treatments, the cultures were transferred to MS medium with 0.06M sucrose and incubated for a further 28d. The survival of shoot buds was monitored every 7d and the final data were recorded after 28d.

The data presented in Table 5.2 indicate that there were significant differences ($p<0.001$) between the effects of the treatments, with the T2 sucrose treatment producing the highest proportion of healthy shoots without callus formation in both genotypes.

Treatments T_6 andT_7 involving a combination of glycerol and sucrose produced the lowest proportion of healthy shoots. There were no significant differences between the responses of the genotypes, although the genotype 865M produced more healthy shoots than the genotype 30MT.

Table 5.1 The effect of plant growth regulators on plant regeneration from shoot buds of two sweet potato genotypes

PGRs treatment	Plant Development (%)							
	865M				30MT			
	Shoot only	Root only	Shoot + Root	Callus*	Shoot only	Root only	Shoot + Root	Callus*
T_1	40	30	30	7	40	27	33	30
T_2	50	10	40	10	43	14	43	10
T_3	7	86	7	17	10	80	10	20
T_4	40	23	37	30	44	23	33	23
T_5	47	23	30	10	40	20	40	13
T_6	37	30	33	20	40	33	27	23
T_7	33	17	50	0	27	20	53	0

n=30 * Cultures producing callus

1. Plant growth regulator (PGRs) treatments:

T_1: MS+ 0.06M sucrose (control) (28d)

T_2: 1μM GA_3 (28)d

T_3: 1μM NAA (28)d

T_4: 1μM BAP (28)d

T_5: 1μM IBA (28)d

T_6: 1μM 2,4-D (28)d

T_7: 1μM GA_3 + 0.02μM NAA

2. Basal medium: MS + 0.06M sucrose

3. Replication: 3 Petri dishes x 10 shoot buds (n=30)

4. Incubation conditions : 25°C / 16h photoperiod ($70\mu Mm^{-2} s^{-1}$ PAR)

Table 5.2. The effect of cryopreservation media on the survival of shoot buds from two sweet potato genotypes (865M and 30MT).

Media treatments	Survival (%)					
	865M			30MT		
	healthy	abnormal	callus*	healthy	abnormal	callus*
T_1	85	5	0	70	0	10
T_2	90	0	0	95	0	0
T_3	75	5	10	80	5	5
T_4	60	5	10	60	5	15
T_5	50	5	5	55	0	10
T_6	30	5	10	20	10	10
T_7	40	10	5	25	10	15

n=30 * Cultures producing callus

1. Media treatment

T_1: MS+ 0.06M sucrose (control) (28d)

T_2: 0.1M sucrose (3d) 0.06M sucrose (28d)

T_3: 0.15M sucrose (3d) 0.06M sucrose (28d)

T_4: 0.4M sucrose (3d) 0.06M sucrose (28d)

T_5: 0.7M sucrose (2d) 0.06M sucrose (28d

T_6: 0.8M sucrose + 1M glycerol (18h) 0.06M sucrose (28d)

T_7: 0.4M sucrose + 2M glycerol (18h) 0.06M sucrose (28d)

5. Basal medium: MS + 1μMGA_3 + 0.02μM NAA

6. Replication: 3 Petri dishes x 10 shoot buds (n=30)

7. Incubation conditions : 25°C / 16h photoperiod (70μMm^{-2} s^{-1} PAR)

5.3: THE EFFECT OF SUCROSE TREATMENTS, EVAPORATIVE DEHYDRATION AND TWO-STEP FREEZING ON THE SURVIVAL OF ENCAPSULATED SHOOT BUDS FROM TWO SWEET POTATO GENOTYPES

As in the previous set of experiments with embryogenic tissues (see Chapter 4.0), once the responses of the shoot buds to various sucrose pre-treatments had been established attention was turned to their responses togel-encapsulation in combination with evaporative dehydration and the two-step freezing process.

Because of the number of treatments (two sucrose treatments x three dehydration times) investigated with each of two genotypes, it was necessary to deal with the individual genotypes in separate experiments. Identical procedures based on the results obtained in Chapter 4 (Tables 4.7 and 4.9) were therefore applied to shoot buds of the two genotypes, with the aim of investigating the effects of the combined sucrose and dehydration treatments on the survival of encapsulated shoot buds. The full procedure for the experiment is outlined in Fig.8.

5.3.1 GENOTYPE 865M

The data presented in Table 5.3 did not indicate any significant differences between the effects of the sucrose treatments T_1 and T_2 on the survival of non-frozen shoot tissue. There were however, significant differences between the effects of the dehydration times ($p<0.001$), with the 3h period showing the highest survival rates if shoots in the non-frozen control. There was no survival among any of the buds subjected to the two-step freezing process.

Table 5.3 Effect of sucrose treatments, evaporative dehydration and two-step freezing on the survival of encapsulated shoot buds in sweet potato genotype 865M

Sucrose treatment	Dehydration (h)	Moisture content (%)	Survival (%)					
			Non-frozen			Two-step freezing		
			Healthy	Abnor-mal	Callus*	Healthy	Abnor-mal	Callus *
T_1	0	72	90	5	5	0	0	0
	3	23	85	5	5	0	0	0
	4	21	80	5	5	0	0	0
	5	20	40	0	5	0	0	0
T_2	0	73	80	5	5	0	0	0
	3	22	90	5	0	0	0	0
	4	21	62	5	0	0	0	0
	5	18	33	0	5	0	0	0

n=21 * Cultures producing callus

1. Sucrose treatment:

Before Dehydration / freezing After dehydration / thawing

T_1: 0.1M(3d):encapsulation:0.4M(2d):0.7M(2d) →0.1M(2d)in dark:0.1M (2d) in light:de-bead:0.1M (28d)

T_2: 0.1M(3d): encapsulation: 0.4M(3d):0.7M(2d):1.0M(2d) →0.1M(2d)in dark:0.1M (2d) in light:de-bead: 0.1M (28d)

2. Basal Medium: MS
3. Replication: 3 Petri dishes x 7 shoot buds(n=21)
4. Freezing Procedure: ambient temperature to 0°C at $10min^{-1}$; 0°C to -40 °C at 0.5 °C min^{-1}, followed by transfer to liquid nitrogen
5. Incubation conditions: 25°C /16h photoperiod (70μM m^{-2} s^{-1} PAR).

5.3.2 GENOTYPE 30MT

The data presented in Table 5.4 indicate that the overall pattern of results obtained with this genotype similar to those obtained with genotype 865M.

5.4: THE EFFECT OF SUCROSE AND GLYCEROL TREATMENTS, EVAPORATIVE DEHYDRATION AND TWO-STEP FREEZING ON THE SURVIVAL OF ENCAPSULATED SHOOT BUDS FROM TWO SWEET POTATO GENOTYPES

Following the studies with sucrose treatments and evaporative dehydration in Section 5.3 showing that an attempt was made with two genotypes (865M and 30MT) to improve survival rates. This utilized a mixture of sucrose and glycerol treatments which was based on those employed by other workers (Matsumoto et al, 1995). In addition of GA_3 and NAA based on the results obtained in Section 5.1, were made to all of the media used in the pre and post-freezing treatments.

For each genotype, the experimental procedure was similar to that outlined in Fig 8 with the following modifications in protocol:

Before dehydration freezing	After freezing/ thawing
T_1	
i) 0.1M sucrose (2d)	i) 0.1M sucrose (2d) in dark
ii) Encapsulation	ii) 0.1M sucrose (2d) in light
iii) 0.15M sucrose (3d)	iii) de-bead
iv) 0.8M sucrose + 1M glycerol	iv) 0.1M sucrose (28d)
T_2	
i) 0.1M sucrose (2d)	i) 0.1M sucrose (2d) in dark
ii) Encapsulation	ii) 0.1M sucrose(2d) in light

iii) 0.15M sucrose (3d)

iv) 0.4M sucrose + 2M glycerol(16h)

iii) de-bead

iv) 0.1M sucrose (28d)

2 Basal medium : MS + 1μM GA_3 + 0.2μM NAA

5.4.1 GENOTYPE 865M

The data presented in Table 5.5 show that there was a low level of survival among frozen shoot buds (maximum survival rate =14%). There was no significant difference between the effects of the sucrose treatment on the survival of frozen shoot buds. However there were significant differences between the effects of the dehydration times ($p< 0.05$), with the 5h treatment (19-27% moisture content) producing the highest survival of shoot buds. With the non-frozen controls, there were again no significant differences between the effects of the sucrose treatments (T_1-T_2). There were significant differences between the effects of the dehydration times, with the 3h treatment producing the highest survival rates of shoot buds.

5.4.2 GENOTYPE 30 MT

The data presented in Table 5.6 show that there was no significant difference between the effects of the sucrose treatments (T_1 and T_2) on the survival of frozen shoot buds. There were significant differences however, between the effects of the dehydration times ($p<0.05$), with the 5h (20-27% moisture content) producing the highest survival rates of shoot buds. With the non-frozen controls, there were significant differences ($p<0.05$) between the effects of the sucrose treatments, with the T_1 producing the highest survival rates of shoot buds. There were also significant differences between the effects of the dehydration times ($p<0.01$), with the 4h treatment producing the highest survival rates.

Table 5.4 The effect of sucrose treatments, evaporative dehydration and two-step freezing on the survival of encapsulated shoot buds in sweet potato genotype 30MT

Sucrose treatment	Dehydration time (h)	Moisture content (%)	Survival (%)					
			Non-frozen			Two-step freezing		
			Healthy	Abnor-mal	Callus*	Healthy	Abnor-mal	Callus *
T_1	0	73	90	0	5	0	0	0
	3	24	95	0	5	0	0	0
	4	21	80	0	5	0	0	0
	5	18	43	0	0	0	0	0
T_2	0	73	95	0	5	0	0	0
	3	25	90	0	5	0	0	0
	4	23	95	0	0	0	0	0
	5	22	43	5	5	0	0	0

n=21 * Cultures producing callus

For experimental details see Table 5.3

Table 5.5. The effect of sucrose and glycerol treatments, evaporative dehydration and two-step freezing on the survival of encapsulated shoot buds from sweet potato genotype 865M

Cryo-protection treatment	Dehydration time (h)	Moisture content (%)	Survival (%)					
			Non-frozen			Two-step freezing		
			Healthy	Abnor-mal	Callus	Healthy	Abnor-mal	Callus
T_1	0	73-74	80	0	5	0	0	0
	3	28-30	90	0	5	0	0	0
	4	27-28	76	0	5	5	0	5
	5	26-27	24	0	5	9	5	5
T_2	0	73	67	14	19	0	0	0
	3	22-32	71	5	10	5	0	0
	4	21-25	85	5	5	0	5	14
	5	19-22	48	0	24	14	5	10

n=21

1. Cryoprotectant treatments:

Before dehydration / freezing	**After dehydration / thawing**
T_1: 0.1Msucrose (2d); encapsulation: 0.15M sucrose(3d): 0.8M sucrose+1M glycerol	0.1M sucrose(2d): indark: 0.1M sucrose (2d): in light: de-bead: 0.1M sucrose (28d)
T_2: 0.1Msucrose (2d); encapsulation: 0.15M sucrose(3d): 0.4M sucrose+2M glycerol	0.1M sucrose(2d): indark: 0.1M sucrose (2d): in light; de-bead: 0.1M sucrose (28d)

2. Basal Medium: MS + 1μM GA_3 + 0.2μM NAA
3. Replication: 3 petridishes x 7 shoot buds (n=21)
4. Freezing Procedure: ambient temperature to 0°C at $10min^{-1}$; 0°C to -40 °C at 0.5 °C min^{-1}, followed by transfer to liquid nitrogen
5. Incubation conditions: 25°C /16h photoperiod (70μM m^{-2} s^{-1} PAR).

Table 5.6. The effect of sucrose and glycerol treatments, evaporative dehydration and two-step freezing on the survival of encapsulated shoot buds from sweet potato genotype 30MT

Cryo-protection treatment	Dehydration time (h)	Moisture content (%)	Survival (%)					
			Non-frozen			Two-step freezing		
			Healthy	Abnor-mal	Callus	Healthy	Abnor-mal	Callus
T_1	0	73	70	5	10	0	0	0
	3	29-30	80	5	10	10	0	5
	4	27-28	90	5	5	5	0	5
	5	26-27	40	5	10	10	5	9
T_2	0	73	86	9	5	0	0	0
	3	25-32	64	10	14	5	5	5
	4	22-25	57	10	9	5	0	10
	5	20-22	19	5	5	10	5	14

n=21

For experimental details see Table 5.5

5.4.3 General conclusion from experiments 5.4.1-5.4.2

1. Low levels of survival for frozen shoot buds in both sucrose/ glycerol treatments were recorded, but there were no significant differences between the effects of the two treatments.

2. The 5h dehydration time gave better results for frozen shoot buds than any other dehydration time.

3. In non-frozen controls, the 4h dehydration time gave better results than any other dehydration time.

5.5 THE EFFECT OF SUCROSE AND GLYCEROL TREATMENTS, EVAPORATIVE DEHYDRATION AND TWO-STEP FREEZING ON THE SURVIVAL OF ENCAPSULATED SHOOT BUDS FROM FOUR SWEET POTATO GENOTYPES

Following the results obtained in the series of experiments 5.4.1-5.4.2 where two genotypes had been subjected to an alteration in cryopreservation protocols involving different sucrose and glycerol and dehydration treatments, a further experiment was carried out in which a slightly modified version of one of the more successful protocols was applied to four genotypes.

The experimental procedure and the cryopreservation treatments (T_1 and T_2) were the same as those used in the previous experiment (see Section 5.4 for details).

In addition, together with a third treatment (T_3) involving minor changes in the post-freezing/ thawing sucrose treatment was applied:

Before dehydration freezing	After freezing/ thawing
T_3	
i) 0.1M sucrose (2d)	i) 0.15M sucrose (2d) in dark
ii) Encapsulation	ii) 0.15M sucrose (1d) in light
iii) 0.15M sucrose (3d)	iii) 0.1M sucrose (1d) in light
iv) 0.4M sucrose + 2M glycerol	iv) de-bead 0.1M sucrose (28d)
	v) 0.1M sucrose (28d)

5.5.1 GENOTYPE 865M

The data presented in Table 5.7 indicate that there were significant differences ($p<0.05$) between the effects of the sucrose/glycerol, with the T_2 producing the highest survival rates of frozen shoot buds. There were also significant differences between the effects of the dehydration times ($p<0.001$), with the 5h (19-24% moisture content) producing the highest survival rates. With the non-frozen controls, there were no significant differences between the effects of the sucrose/glycerol treatments on the survival rates of shoot buds. There were however significant differences between the effects of the dehydration times ($p<0.001$), with the 0h treatment producing the highest survival rates of shoot buds.

5.5.2 GENOTYPE 1023M

The data presented in Table 5.8 indicate that there were significant differences ($p<0.05$) between the effects of the sucrose/glycerol, with the T_2 cryoprotection treatment producing the highest survival rates of frozen shoot buds. There were also significant differences between the effects of the dehydration times ($p<0.001$), with the 5h dehydration period (21-27% moisture content) producing the highest survival rates. With the non-frozen controls, there were no significant differences between the effects of

Table 5.7. The effect of sucrose and glycerol treatments, evaporative dehydration and two-step freezing on the survival of encapsulated shoot buds from sweet potato genotype 865M

Cryo-protection treatment	Dehydration time (h)	Moisture content (%)	Survival (%)					
			Non-frozen			Two-step freezing		
			Healthy	Abnor-mal	Callus	Healthy	Abnor-mal	Callus
T_1	0	73-74	81	0	5	0	0	0
	3	22-27	90	5	0	0	0	0
	4	20-24	85	0	0	5	0	5
	5	20-23	43	5	5	14	5	10
T_2	0	72-73	71	5	5	0	0	0
	3	21-25	62	5	0	14	0	5
	4	21	81	5	5	24	0	19
	5	19-20	52	0	10	33	5	10
T_3	0	73-74	76	0	10	0	0	0
	3	28-32	57	5	5	0	0	0
	4	25-27	86	5	10	19	0	5
	5	22-24	48	5	14	5	5	14

n=21

1. Cryoprotectant treatments:

	Before dehydration / freezing	**After dehydration / thawing**
T_1	0.1Msucrose(2d); encapsulation; 0.15M sucrose(3d); 0.8M sucrose+1M glycerol (18h)	0.1M sucrose(2d); in dark; 0.1M sucrose (2d); in light; de-bead; 0.1M sucrose (28d)
T_2	0.1Msucrose(2d); encapsulation; 0.15M sucrose(3d); 0.4M sucrose+2M glycerol (18h)	0.1M sucrose(2d); indark; 0.1M sucrose (2d); in light; de-bead; 0.1M sucrose (28d)
T_3	0.1Msucrose(2d); encapsulation; 0.15M sucrose(3d); 0.4M sucrose+2M glycerol (18h)	0.15M sucrose(2d); indark; 0.15M sucrose (1d); in light; 0.1M suc; de-bead; 0.1M sucrose (28d)

4. Basal Medium: MS + 1μM GA_3 + 0.2μM NAA
5. Replication: 3 petridishes x 7 shoot buds (n=21)
6. Freezing Procedure: ambient temperature to 0°C at $10min^{-1}$; 0°C to -40 °C at 0.5 °C min^{-1}, followed by transfer to liquid nitrogen
7. Incubation conditions: 25°C /16h photoperiod (70μM m^{-2} s^{-1} PAR).

Table 5.8. The effect of sucrose and glycerol treatments, evaporative dehydration and two-step freezing on the survival of encapsulated shoot buds from sweet potato genotype 1023M

Cryo-protection treatment	Dehydration time (h)	Moisture content (%)	Survival (%)					
			Non-frozen			Two-step freezing		
			Healthy	Abnor-mal	Callus	Healthy	Abnor-mal	Callus
T_1	0	72-73	80	0	5	0	0	0
	3	30-32	78	5	5	0	0	0
	4	25-27	71	0	5	5	0	0
	5	21-24	43	0	10	0	0	9
T_2	0	73-74	70	5	10	0	0	0
	3	32-33	80	0	5	0	5	5
	4	25-24	91	0	0	5	0	0
	5	22-24	62	0	10	10	5	10
T_3	0	74	76	5	5	0	0	0
	3	33-34	81	0	10	5	5	0
	4	28-30	90	0	0	0	0	5
	5	25-27	52	0	9	5	0	10

n=21

For experimental details see Table 5.7

the sucrose/ glycerol treatments on the survival rates of shoot buds. There were significant differences between the effects of the dehydration times ($p<0.001$), with the 4h treatment producing the highest survival rates of shoot buds.

5.5.3 GENOTYPE 30MT

The data presented in Table 5.9 indicate that there were significant differences ($p<0.05$) between the effects of the sucrose/glycerol, with the T_2 producing the highest survival rates of frozen shoot buds. There were also significant differences between the effects of the dehydration times ($p<0.001$), with the 5h (20–27% moisture content) producing the highest survival rates. With the non-frozen controls, there were no significant differences between the effects of the sucrose/glycerol treatments on the survival rates of shoot buds. There were significant differences between the effects of the dehydration times ($p<0.001$), with the 0h treatment producing the highest survival rates.

5.5.4 GENOTYPE 132M

The data presented in Table 5.10 indicate that there were significant differences ($p<0.05$) between the effects of the sucrose/glycerol, with T_2 producing the highest survival rates of frozen shoot buds. There were also significant differences between the effects of the dehydration times ($p<0.001$), with the 5h producing the highest survival rates. With the non-frozen controls, there were no significant differences between the effects of the sucrose/glycerol treatments on the survival rates of shoot buds. There were significant differences between the effects of the dehydration times ($p<0.001$), with the 4h treatment producing the highest survival rates.

Table 5.9. The effect of sucrose and glycerol treatments, evaporative dehydration and two-step freezing on the survival of encapsulated shoot buds from sweet potato genotype 30MT

Cryo-protection treatment	Dehydration time (h)	Moisture content (%)	Survival (%)					
			Non-frozen			Two-step freezing		
			Healthy	Abnor-mal	Callus	Healthy	Abnor-mal	Callus
T_1	0	73-74	76	5	5	0	0	0
	3	28-30	81	0	0	0	0	0
	4	27-28	90	0	5	5	0	10
	5	26-27	50	0	10	10	0	14
T_2	0	72-73	81	5	5	0	0	0
	3	25-32	76	0	10	0	0	0
	4	22-25	57	5	5	5	0	9
	5	20-22	24	5	10	19	0	23
T_3	0	73	86	0	5	0	0	0
	3	26-30	67	0	14	0	0	5
	4	25-26	57	5	14	10	4	10
	5	20-24	19	10	5	0	0	0

n=21

For experimental details see Table 5.7

Table 5.10. The effect of sucrose and glycerol treatments, evaporative dehydration and two-step freezing on the survival of encapsulated shoot buds from sweet potato genotype 132M

Cryo-protection treatment	Dehydration time (h)	Moisture content (%)	Survival (%)					
			Non-frozen			Two-step freezing		
			Healthy	Abnor-mal	Callus	Healthy	Abnor-mal	Callus
T_1	0	65-72	90	0	5	0	0	0
	3	22-25	95	0	0	0	0	5
	4	21-22	81	0	5	5	0	10
	5	18-20	38	5	5	10	0	5
T_2	0	72-73	71	5	10	0	0	0
	3	24-25	62	5	5	0	0	0
	4	23-24	86	0	10	5	5	9
	5	22-23	43	0	5	14	5	5
T_3	0	72-73	81	0	5	0	0	0
	3	25	71	5	0	0	0	0
	4	23-25	90	0	5	10	0	5
	5	20-22	52	0	5	0	10	5

n=21

For experimental details see Table 5.7

5.5.5 GENERAL CONCLUSION FROM EXPERIMETS 5.5.1-5.5.4

1. The T_2 sucrose/glycerol treatment with all genotypes showed the best results for the survival of frozen shoot buds.
2. Genotype 865M showed the highest survival rates for frozen shoot buds, with values which were higher than in the previous experiment (5.4).
3. The 5h dehydration time showed the best results for the survival of frozen shoot buds.
4. In the non-frozen controls, sucrose/ glycerol treatment T_1 showed the best results for survival of shoot buds with all genotypes.
5. The 3h dehydration time in all sucrose/ glycerol treatments showed the best survival rates for non-frozen shoot buds.

5.6 THE EFFECT OF BUD SIZE, EVAPORATIVE DEHYDRATION AND TWO-STEP FREEZING ON THE SURVIVAL OF ENCAPSULATED SHOOT BUDS FROM TWO SWEET POTATO GENOTYPES

Because of the improved survival rates obtained in 5.5 with cryoprotection treatment T_2 and genotype 865M, the same treatment/ genotype combination was used in this experiment, together with a second genotype (30MT) to investigate the responses of shoot buds of different sizes to the freezing procedure.

The experimental procedure was similar to that outlined in Fig. 8 , except that the MS medium used in all of the cryoprotectant mixture was supplemented with 1μM GA_3 + 0.2 μM NAA. The sucrose/ glycerol treatment (T_2) was the same as that used in the previous experiment (see Table 5.11 for details). Two buds sizes (0.2-0.5mm and 0.5-1.0mm), taken from 28d old plants cultured on MS medium with 0.06M sucrose, were selected for investigation.

5.6.1 GENOTYPE 865M

The data presented in Table 5.11 show that there were significant differences ($p<0.05$) between the effects of the size on the responses of the frozen buds, with the smaller size (0.2-0.5mm) producing the higher survival rates. There were also significant differences between the effects of the dehydration times ($p<0.001$), with the 5h (25-28% moisture content) producing the highest survival of frozen shoot buds. With the non-frozen controls, there were no significant differences between the response of buds of different sizes. There were, however, significant differences between the effects of the dehydration times ($p<0.01$), with the 0h dehydration producing the highest survival rates.

5.6.2 GENOTYPE 30MT

The data presented in Table 5.12 essentially show the same pattern of results as obtained with genotype 865M, although the overall survival rates were somewhat lower and survival of frozen buds was only obtained following the 5h dehydration period (26-33% moisture content).

Therefore, smaller buds were routinely used in the subsequent experiments.

Table 5.11. The effect of sucrose and glycerol treatments, evaporative dehydration and two-step freezing on the survival of encapsulated shoot buds from sweet potato genotype 865M

Bud size (mm)	Dehydration time (h)	Moisture content (%)	Survival (%)					
			Non-frozen			Two-step freezing		
			Healthy	Abnor-mal	Callus	Healthy	Abnor-mal	Callus
0.2-0.5	0	73-74	80	5	5	0	0	0
	3	30-32	76	0	5	0	5	10
	4	27-30	71	4	5	14	5	10
	5	25-26	62	4	10	19	0	5
0.5-1.0	0	73	90	0	5	0	0	0
	3	32-33	81	5	0	0	5	0
	4	28-30	61	5	10	5	0	5
	5	26-28	52	5	5	10	0	9

n=21

1. Cryoprotectant treatment:

Before Dehydration / freezing	After dehydration / thawing
0.1Msucrose(2d): encapsulation: 0.15M sucrose(3d): 0.4M sucrose+2M glycerol (18h).	0.1M sucrose(2d): indark: 0.1M sucrose (2d): in light: de-bead: 0.1M sucrose (28d)

2. Basal Medium: MS + 1μM GA_3 + 0.2μM NAA
3. Replication: 3 Petri dishes x 7 shoot buds (n=21)
4. Freezing Procedure: ambient temperature to 0°C at $10min^{-1}$; 0°C to -40 °C at 0.5 °C min^{-1}, followed by transfer to liquid nitrogen
5. Incubation conditions: 25°C /16h photoperiod (70μM m^{-2} s^{-1} PAR).

Table 5.12. The effect of sucrose and glycerol treatments, evaporative dehydration and two-step freezing on the survival of encapsulated shoot buds from sweet potato genotype 30MT

Bud size (mm)	Dehydration time (h)	Moisture content (%)	Survival (%)					
			Non-frozen			Two-step freezing		
			Healthy	Abnor-mal	Callus	Healthy	Abnor-mal	Callus
0.2-0.5	0	72-73	71	14	14	0	0	0
	3	37-1	24	14	19	0	0	0
	4	34-35	43	14	19	0	0	14
	5	32-33	29	14	10	14	0	29
0.5-1.0	0	72-73	80	5	10	0	0	0
	3	30-42	67	14	19	0	0	10
	4	29-33	52	10	14	0	5	10
	5	26-32	33	14	19	5	0	9

n=21

For experimental details see Table 5.11

5.7 THE EFFECT OF SUCROSE/ GLYCEROL AND SUCROSE/ MANNITOL TREATMENTS, EVAPORATIVE DEHYDRATION AND TWO-STEP FREEZING ON THE SURVIVAL OF ENCAPSULATED SHOOT BUDS IN GENOTYPE 865M

In this experiment, two further cryopreservation treatments were investigated: T_1 involving a pre-freezing sequence in which a mixture of 0.4M sucrose and 1M mannitol was used for the final stage, T_2 in which glycerol (1M) alone was used in the final stage. The experimental procedure was similar to that outlined in Fig 8, except that the MS medium used in all of the cryoprotectant mixture was supplemented with 1μM GA_3 + 0.2 μM NAA. The following cryopreservation treatments were employed:

Before dehydration freezing	After freezing/ thawing
T_1	
i) 0.1M sucrose (2d)	i) 0.1M sucrose (2d) in dark
ii) Encapsulation	ii) 0.1M sucrose (2d) in light
iii) 0.15M sucrose (3d)	iii) de-bead
iv) 0.4M sucrose + 1M mannitol (18h)	iv) 0.1M sucrose (28d)
T_2	
i) 0.1M sucrose (2d)	i) 0.1M sucrose (2d) in dark
ii) Encapsulation	ii) 0.1M sucrose (2d) in light
iii) 0.15M sucrose (3d)	iii) de-bead
iv) 0.7M sucrose (1d)	iv) 0.1M sucrose (28d)
v)1M glycerol (18h)	

The data presented in Table 5.13 show that there was no survival of frozen buds that had been subjected to either cryoprotectant treatment. There was however, survival among

the non-frozen buds and the analysis of deviance (appendix 5.9) show that there were significant differences between the effects of dehydration times ($p<0.01$). The 4h period (26-27% moisture content) showed the highest survival rates, but not between the effects of two cryoprotection treatments.

5.8 THE EFFECT OF SUCROSE/ GLYCEROL/ MANNITOL/ PROLINE AND SUCROSE AND GLYCEROLTREATMENTS, EVAPORATIVE DEHYDRATION AND TWO-STEP FREEZING ON THE SURVIVAL OF ENCAPSULATED SHOOT BUDS IN GENOTYPE 30MT

In this experiment, a further compound, proline, which has been used in a pre-freezing cryoprotection sequence of treatments was introduced. Comparisons were made with a sucrose/ glycerol sequence using genotype 30MT.

The experimental procedure was similar to that used in 5.7 and the following cryopreservation treatments were employed:

Before dehydration freezing	After freezing/ thawing
T_1	
i) 0.1M sucrose (2d)	i) 0.1M sucrose (2d) in dark
ii) Encapsulation	ii) 0.1M sucrose (2d) in light
iii) 0.15M sucrose (3d)	iii) de-bead
iv) 1.0M sucrose + 0.5M glycerol + 39g/l mannitol + 5g/l proline	iv) 0.1M sucrose (28d)
T_2	
i) 0.1M sucrose (2d)	i) 0.1M sucrose (2d) in dark
ii) Encapsulation	ii) 0.1M sucrose (2d) in light

iii) 0.15M sucrose (3d)

iv) 0.7M sucrose (1d)

v)1M glycerol (18h)

iii) de-bead

iv) 0.1M sucrose (28d)

The data presented in Table 5.14 show that there was no survival of frozen shoot buds subjected to either of the cryoprotection treatments. There was however, survival of non-frozen buds and the accumulated analysis of deviance (appendix 5.10) did not show any significant differences between the effects of the sucrose/ glycerol/ mannitol/ proline and the sucrose/ glycerol treatments (T_1 and T_2). There were however significant differences between the effects of the dehydration times ($p<0.01$), with the 4h period showing the highest survival rates.

5.9 THE EFFECTS OF VARIOUS TREATMENTS WITH PVS2 CRYOPROTECTANT MIXTURES AND RAPID FREEZING ON THE SURVIVAL OF ENCAPSULATED SHOOT BUDS FROM TWO SWEET POTATO GENOTYPES

Following the previous investigations into cryopreservation of shoot buds, which had all involved protocols on a two-step freezing procedure, a small series of experiments were carried out with protocol involving rapid freezing. Additionally, in this experiment, the effects of a cryoprotectant mixture containing a combination (PVS2) of glycerol, ethylene glycol and DMSO (see section 2.6.1 for details) were investigated with two genotypes (865M and Nem).

The experimental procedure is outlined in Fig. 9 and used for each genotype described under Table 5.15.

Table 5.13. The effect of sucrose/ glycerol and sucrose/ mannitol cryoprotection treatments, evaporative dehydration and two-step freezing on the survival of encapsulated shoot buds from sweet potato genotype 865M

Cryo-protection treatment	Dehydration time (h)	Moisture content (%)	Survival (%)					
			Non-frozen			Two-step freezing		
			Healthy	Abnor-mal	Callus	Healthy	Abnor-mal	Callus
T_1	0	68-70	86	14	0	0	0	0
	3	28-31	81	14	5	0	0	0
	4	26-27	90	10	0	0	0	0
	5	26	71	15	14	0	0	0
T_2	0	73-74	81	14	5	0	0	0
	3	27-28	90	5	0	0	0	0
	4	26-27	90	10	0	0	0	0
	5	24-25	67	14	0	0	0	0

n=21

1. Cryoprotectant treatments:

	Before dehydration / freezing	**After dehydration / thawing**
T_1:	0.1M(2d)sucrose: encapsulation: 0.15M sucrose(3d): 0.4M sucrose+1M mannitol (18h)	0.1M sucrose(2d): indark: 0.1M sucrose (2d): in light: de-bead: 0.1M sucrose (28d)
T_2:	0.1M(2d)sucrose: encapsulation: 0.15M sucrose(3d): 0.7M sucrose (1d): 1M glycerol (18h)	0.1M sucrose(2d): indark: 0.1M sucrose (2d): in light: de-bead: 0.1M sucrose (28d)

2. Basal Medium: MS + 1μM GA_3 + 0.02μM NAA
3. Replication: 3 petridishes x 7 shoot buds (n=21)
4. Freezing Procedure: ambient temperature to 0°C at $10min^{-1}$; 0°C to -40 °C at 0.5 °C min^{-1}, followed by transfer to liquid nitrogen
5. Incubation conditions: 25°C /16h photoperiod (70μM m^{-2} s^{-1} PAR).

Table 5.14. The effect of sucrose/ glycerol and sucrose/ mannitol cryoprotection treatments, evaporative dehydration and two-step freezing on the survival of encapsulated shoot buds from sweet potato genotype 30MT

Cryo-protection treatment	Dehydration time (h)	Moisture content (%)	Survival (%)					
			Non-frozen			Two-step freezing		
			Healthy	Abnor-mal	Callus	Healthy	Abnor-mal	Callus
T_1	0	60-62	85	10	5	0	0	0
	3	28-29	81	5	10	0	0	0
	4	27	90	0	5	0	0	0
	5	25-26	76	14	5	0	0	0
T_2	0	70-72	82	0	18	0	0	0
	3	35-36	93	7	0	0	0	0
	4	31-32	90	5	5	0	0	0
	5	27-29	64	7	29	0	0	0

n=21

1. Cryoprotectant treatments:

	Before dehydration / freezing	**After dehydration / thawing**
T_1:	0.1Msucrose (2d); encapsulation; 0.15M sucrose(3d); 1.0Msucrose+0.5M glycerol + 39g/l mannitol + 5g/l proline (18h)	0.1M sucrose(2d); indark; 0.1M sucrose (2d); in light; de-bead; 0.1M sucrose (28d)
T_2:	0.1M(2d)sucrose; encapsulation; 0.15M sucrose(3d); 0.7M sucrose (1d); 1M glycerol (18h)	0.1M sucrose(2d); indark; 0.1M sucrose (2d); in light; de-bead; 0.1M sucrose (28d)

2. Basal Medium: MS + 1μM GA_3 + 0.2μM NAA
3. Replication: 3 Petri dishes x 7 shoot buds (n=21)
4. Freezing Procedure: ambient temperature to 0°C at $10min^{-1}$; 0°C to -40 °C at 0.5 °C min^{-1}, followed by transfer to liquid nitrogen
5. Incubation conditions: 25°C /16h photoperiod (70μM m^{-2} s^{-1} PAR.

Table 5.15. The effect of various treatments with PVS2 cryoprotectant mixtures and rapid freezing in the survival of encapsulated shoot buds from genotype 865M

PVS2* conc. (%)	Exposure to PVS2 (min)	Survival (%)					
		Non-frozen			Rapid freezing		
		Healthy	Abnormal	Callus	Healthy	Abnormal	Callus
100	2	57	5	9	0	0	0
80	5	76	5	14	0	0	0
60	10	86	5	9	0	0	0
40	15	90	5	5	0	0	0
20	20	100	0	0	0	0	0

n=30

Pre-freezing treatment

0.1M (2d); encapsulation; 0.15M(3d); 0.8M + 1M glycerol (18h); transfer to PVS2 solution (20-100%) for 2-20min.

Post-freezing treatment

washed in MS + 1.2M (twice) + 0.1M sucrose in dark; 0.1Msucrose (2d) in light; de-bead; 0.1M sucrose (28d).

* PVS2 solution was diluted with MS + 0.4M sucrose

3. Basal Medium: MS + 1μM GA_3 + 0.2μM NAA
4. PVS2 solution:

   -Glycerol (30%v/v)

   -Ethylene glycol (15% v/v)

   -DMSO (15% v/v)
5. Incubation conditions: 25°C /16h photoperiod (70μM m^{-2} s^{-1} PAR).
6. Freezing procedure: samples were directly immersed in LN_2 for 1h.

5.9.1. GENOTYPE 865M

The data presented in Table 5.15 show that there was no survival of any of the buds which had been subjected to the rapid-freezing process. There was however survival of non-frozen buds and the accumulated analysis of deviance (not shown) show that there were significant differences ($p<0.01$) between the effects of the PVS2 treatments, with the 20% level producing the highest survival rates.

5.9.2. GENOTYPE NEM

Essentially the same pattern of results was obtained with genotype Nem as with genotype 865M.

5.10 THE EFFECT OF VARIOUS TREATMENTS WITH PVS2 AND RAPID FREEZING ON THE SURVIVAL OF NON-ENCAPSULATED SHOOT BUDS FROM TWO SWEET POTATO GENOTYPES

In the previous experiment, a rapid freezing process that had been used with some success with embryogenic tissue (see section 4.12) was used without success with the shoot buds. Recognizing that rapid freezing techniques may not be suitable for use with encapsulated tissue because of the bulk of the gel-bead, the previous experiment was repeated using the same procedure (see Fig. 9), except for the omission of the encapsulation stage.

5.10.1 GENOTYPE 865M

The data presented in Table 5.17 show that there was no survival of frozen buds following any of the treatments. There was however, survival of non-frozen buds and the accumulated analysis of deviance (not shown) shows that there were significant

Table 5.16. The effect of various treatments with PVS2 cryoprotectant mixtures and rapid freezing on the survival of encapsulated shoot buds in genotype Nem

PVS2 conc. (%)	Exposure to PVS2 (min)	Survival (%)					
		Non-frozen			Rapid freezing		
		Healthy	Abnormal	Callus	Healthy	Abnormal	Callus
100	2	70	5	10	0	0	0
80	5	80	10	5	0	0	0
60	10	90	5	0	0	0	0
40	15	90	0	0	0	0	0
20	20	95	0	5	0	0	0

n=30

For experimental details see Table 5.15

Table 5.17. The effect of various treatments with PVS2 cryoprotectant mixtures and rapid freezing on the survival of non-encapsulated shoot buds in genotype 865M

PVS2* conc. (%)	Exposure to PVS2 (min)	Survival (%)					
		Non-frozen			Rapid freezing		
		Healthy	Abnormal	Callus	Healthy	Abnormal	Callus
100	2	43	5	5	0	0	0
80	5	50	10	0	0	0	0
60	10	76	5	5	0	0	0
40	15	81	5	5	0	0	0
20	20	86	4	10	0	0	0

n=30

1. Pre-freezing treatment

0.1M (2d); 0.15M(3d); 0.8M + 1M glycerol (18h); before transfer to PVS2 solution (20-100%) for 2-20min.

Post-freezing treatment

washed in MS + 1.2M (twice) + 0.1M (2d); in dark; 0.1M (28d)

* PVS2 solution was diluted with MS + 0.4M sucrose

2. Basal Medium: MS + 1μM GA_3 + 0.02μM NAA

3. PVS2 solution:

-Glycerol (30%v/v)

-Ethylene glycol (15% v/v)

-DMSO (15% v/v)

4. Replication: 3 petridishes x 10 shoot buds (n=30)

5. Incubation conditions: 25°C /16h photoperiod (70μM m^{-2} s^{-1} PAR).

6. Freezing procedure: samples were directly immersed in LN_2 for 1h.

differences ($p<0.001$) between the effects of the PVS2 treatments, with the 20% level producing the highest survival rates.

5.10.2 GENOTYPE NEM

The pattern of results obtained with this genotype was essentially the same as that obtained with genotype 865M, although the overall survival rates were somewhat lower.

5.11 THE EFFECT OF SUCROSE, EVAPORATIVE DEHYDRATION WITH SILICA GEL AND RAPID FREEZING ON THE SURVIVAL OF NON-ENCAPSULATED NODAL SEGMENTS IN SWEET POTATO GENOTYPE 1023M

All of the previous experiments in this chapter had involved shoot buds, which had been excised from their surrounding tissue before being subjected to the various cryopreservation procedures. In this final experiment with a single genotype (1023M) an alternative approach was taken in which intact nodal segments, containing the axillary buds, were taken through a procedure involving evaporative dehydration in the presence of silica-gel, followed by rapid freezing. This was attempted because it was considered that the excised buds would have been too small and delicate to have survived the evaporative dehydration procedure.

The experimental procedure was similar to that used in Fig 8 with following changes:

1. Sucrose treatment

Before dehydration freezing	After freezing/ thawing
0.1M(1d); 0.7M(2d)	0.1M(2d); in dark; 0.1m(28d)

2. Experimental tissue: nodal segment (5mm long)

7. Dehydration times in presence of silica-gel (10 nodal segments in petridish containing 15g silica-gel - 5-8h.
8. Freezing Procedure: Rapid-freezing (direct immersion in liquid nitrogen for 1h).

The data presented in Table 5.19 show that there was no survival of any of the frozen nodal segments. There was, however, survival of non-frozen tissue, shows that there were significant differences ($p<0.001$) between the effects of the dehydration times, with non-dehydrated tissue (0h) producing the highest survival rates.

Table 5.18. The effect of various treatments with PVS2 cryoprotectant mixtures and rapid freezing on the survival of non-encapsulated shoot buds in genotype Nem

PVS2 conc. (%)	Exposure to PVS2 (min)	Survival (%)					
		Non-frozen			Rapid freezing		
		Healthy	Abnormal	Callus	Healthy	Abnormal	Callus
100	2	14	0	5	0	0	0
80	5	43	10	9	0	0	0
60	10	62	9	10	0	0	0
40	15	50	10	20	0	0	0
20	20	60	10	20	0	0	0

n=30

For experimental details see Table 5.17.

Table 5.19. The effect of sucrose and evaporative dehydration with silica-gel and rapid freezing on the survival of nodal segments in sweet potato genotype 1023M

Dehydration (h)	Moisture content (%)	Survival (%)					
		Non-frozen			Rapid freezing		
		Healthy	Abnormal	Callus	Healthy	Abnormal	Callus
0	73-74	80	5	10	0	0	0
5	33-34	20	10	4	0	0	0
6	30-32	20	0	0	0	0	0
7	23-25	40	10	10	0	0	0
8	23	14	5	0	0	0	0

n=21

1. Sucrose treatment

Before freezing	After freezing
0.1M (1d); 0.7M(2d)	0.1M (2d); in dark; 0.1M (28d)

2. Basal Medium: MS + 1μM GA_3 + 0.2μM NAA
3. Size of nodal segment = 5.0mm in length
4. Silica-gel/ Petri dish = 15g
5. Replication: 3 petridishes x 10 shoot buds (n=21)
6. Incubation conditions: 25°C /16h photoperiod (70μM m^{-2} s^{-1} PAR). Immediately after thawing, cultures were incubated at 25°C in the dark for 2d.
7. Freezing procedure: samples were directly immersed in LN_2 for 1h.

Fig. 8 Cryopreservation protocol for shoot buds

Three batches of 10 shoot buds were selected for each experimental interaction (2 genotypes x 4 dehydration times x frozen and unfrozen treatments). Each batch of shoot buds were subjected to the following procedure:

a. Buds were placed in a petri dish containing 10ml MS medium with 0.1M sucrose for 3d in the light (16 photoperiod) at 25°C.

b. Each bud was encapsulated in an alginate bead (see section 2.6.2).

c. The beads were transferred through a sequence of sucrose treatments as indicated under Table 5.3 (T_1-T_2). For each stage in the sequence the batch was placed in a petri dish containing 10ml MS medium and the appropriate concentration of sucrose.

d. Beads were removed from petri dishes and dehydrated for the appropriate time (3-5h); 3 beads were used to determine moisture content

e. The remaining 7 beads were subjected to a two-step freezing procedure (except for the unfrozen controls): beads transferred to a 2ml cryovial and slow-cooled to 0°C at $10min^{-1}$ and from 0°C to -40°C at $0.5min^{-1}$, followed by rapid cooling by direct immersion in LN_2.

f. After storage in LN_2 for 1h, the beads were thawed rapidly by transfer of the cryovials to a water bath at 40°C for 2m.

g. The beads were transferred to a petri dish containing MS medium with 0.1M sucrose for 2d in the dark followed by 2d in light (16h photoperiod) at 25°C.

h. Shoot buds were removed from the beads (de-beaded).

i. Shoot buds were transferred to a petridish containing MS medium and 0.1M sucrose for 28d in the light (16h photoperiod) at 25°C.

j. Survival data were recorded.

Fig. 9. Cryopreservation protocol for shoot buds

Three batches of 10 shoot buds were selected for each experimental interaction (2 genotypes x 5 PVS2 treatments x frozen and unfrozen treatments). Each batch of shoot buds were subjected to the following procedure:

a. Buds were placed in a petri dish containing 10ml MS medium supplemented 1μM GA_3 + 0.02μM NAA and 0.1M sucrose for 2d in the light (16 photoperiod) at 25°C.

b. Each bud was encapsulated in an alginate bead (see Chapter 2, Section 2.6.2).

c. Beads were transferred to a petridish containing MS medium + 1μM GA_3 + 0.02μM NAA and 0.15M sucrose for 3d in the light and then to MS medium + 1μM GA_3 + 0.02μM NAA and 0.8M and glycerol for 18h in the light (16 photoperiod) at 25°C.

d. Beads were transferred to another petridish containing .. ml of one of the PVS2 treatments (100, 80, 60, 40 or 20%) for the appropriate exposure time (2,5,10,15 or 20min), respectively. After storage in LN_2 for 1h, the beads were thawed rapidly by transfer of the cryovials to a water bath at 40°C for 2m.

e. The beads were subjected to a rapid cooling procedure (except for the unfrozen controls): beads transferred to 2ml cryovials containing the appropriate PVS2 solution and directly immersed into LN_2.

f. After storage in LN_2 for 1h, the beads were thawed rapidly by transfer of the cryovials to a water bath at 40°C for 2m.

g. Immediately after thawing the beads were washed twice with MS medium supplemented with 1.2M sucrose.

h. The beads were transferred to a petridish containing MS medium supplemented with + 1μM GA_3 + 0.02μM NAA and 0.1M sucrose for 2d in the dark, followed by 2d in the light (16h photoperiod) at 25°C.

i. Shoot buds were removed from the beads

j. Shoot buds were transferred to a petridish containing MS medium supplemented with 1μM GA_3 + 0.02μM NAA and 0.1M sucrose for 28d in the light (16h photoperiod) at 25°C.

k. Survival data were recorded after 28d

5.12: DISCUSSION

The third objective of this study was to develop techniques for the cryopreservation of sweet potato shoot buds that could be used with a wide range of genotypes in genetic conservation programmes. Early studies on the cryopreservation of sweet potato cell suspension cultures had met with some success (see section 3.2.1), but it had been recognised in subsequent work that shoot meristems from this species are recalcitrant with some of the techniques that had been successful with other species such as potato (Grout and Henshaw, 1978; Kartha, 1985; IBPGR, 1988). The aim here, therefore was to investigate the response of shoot buds from a range of sweet potato genotypes to the newer cryopreservation techniques that had been applied with some success to embryogenic tissues of sweet potato in the earlier parts of this programme (see section 4.7).

Since it can be assumed that shoot buds, or any other tissue, are in a highly stressed state during the early stages of recovery following cryopreservation, it is important that growth conditions should, as far as possible, be optimised. It has to be recognised, that such conditions established with normal tissues may not be entirely relevant to the requirements of stressed tissues following freezing and thawing, but it seems logical in the stages of a cryopreservation investigation to provide the tissue with the best chances of survival and growth. It is only when post-thaw survival has been achieved, that it becomes possible to address more directly the question of the optimisation of post-thaw growth conditions.

Initially, therefore, the effects on the growth and development of shoot buds of a number of plant growth regulators (GA_3, NAA, BAP, IBA, 2,4-D), individually or in combination

were investigated with two sweet potato genotypes. It was found that there were no significant differences between the effects on plant-regeneration of the various growth regulators applied alone except that NAA showed an inhibitory effect on shoot growth. In a qualitative sense, GA3 performed well since plant regeneration was associated with little callus formation, whereas 2,4-D and BAP both tended to promote callus formation. A combination of 1 μM GA3 and 0.2 μM NAA, on the other hand, was found to be very effective in terms of plantlet regeneration, with minimum production of callus tissue. The latter feature is important because the production of genetically unstable callus tissue, which could give rise to genetically variant adventitious shoots is not compatible with the aims of genetic conservation. On the basis of this investigation, the basal medium used in all of the subsequent cryopreservation experiments with shoot buds consisted of MS supplemented with 1 μM GA3 + 0.2 μM NAA and sucrose (see section 5.2 – 5.8).

In previously published work concerning sweet potato, Gunckel et al (1972) were able to produce shoots from two of these genotypes yellow Jersey and Continental by use of a combination of Coconut milk, 40 $mg.l^{-1}$ adenine, 11 $mg.l^{-1}$ GA3 and 1 $mg.l^{-1}$ IAA or a mixture of containing 1 $mg.l^{-1}$ kinetin, 1 $mg.l^{-1}$ GA3 and 1 $mg.l^{-1}$ 2,4-D. Litz and Conover (1978) propagated two varieties of sweet potato (White Star and PI 315343), using explants from shoot-tips and axillary buds grown on MS medium containing BAP, IAA, Kinetin and activated charcoal. Optimal shoot regeneration from White Star explants was induced by BAP (4.4 μM) and from PI 315343 using a mixture of Kinetin (4.6 μM) and IAA (5.7 μM). These have been no previous reports of the use of a combination of the growth regulators GA3 and NAA for the successful production of plantlets from sweet potato shoot meristems with minimum callus formation.

On the basis of work reported by other workers concerning the cryopreservation of shoot buds of species such as pea, maize and pink lily (*Lilium Japomicum, T.*) (see sections 1.3.1.2. and 1.3.3 for details) and the results obtained in section 4.1 with the sweet potato embryogenic tissues, it was decided to investigate, initially, the effects of a range of sucrose concentrations (0.1 M – 0.7 M for 2 – 3 days exposure) alone or in combination with glycerol (0.4 M sucrose + 2 M glycerol for 18 h exposure or 0.8 M sucrose plus 1 M glycerol for 18 exposure) on the survival of unfrozen shoot buds of two sweet potato genotypes (see section 5.2).

The results of this work indicated that in general, these were no significant differences between the effects of the different sucrose treatments on the survival of shoot buds. Although 0.1M sucrose concentration gave the best results in terms of the production of healthy shoots (90 – 95%) without any callus formation. On increasing the sucrose level to 0.7 M. the survival rates of the shoot buds fell to 50 – 55%. These were however, significant differences between the effects on shoot growth and development of sucrose alone and in combination with glycerol. Survival rates of 40% and 30%, respectively, were recorded for shoot buds cultured on a mixtures of 0.4 M sucrose and 2 M glycerol or 0.8 M sucrose and 1 M glycerol for 18h prior to transfer to MS medium containing 0.1 M sucrose.

No work has been reported concerning the effects of sucrose/glycerol treatments on shoot formation from axillary buds of sweet potato. Recently however, a mixture of 2 M glycerol plus 0.4 M sucrose was reported to have been very effective in inducing dehydration tolerance for nuceller cells of orange (Sakai et al, 1991) and Wasabi and Lily (Matsumoto et al, 1995a; 1995b).

Following these preliminary studies with the sweet potato shoot buds, it was decided to proceed with the cryopreservation studies. On the basis of the results obtained with the embryogenic tissues from eight potato genotypes in section 4.7, two of the more successful cryopreservation protocols were applied to shoot buds of two sweet potato genotypes (see section 5.3). These protocols involved the use of sucrose treatments, evaporative dehydration and two-step freezing with encapsulated shoot buds. A further series of experiments was carried out with similar procedures except that the cryoprotectant treatments involved combinations of sucrose and glycerol (see section 5.4). Following results obtained in section 5.4, a further experiments with four sweet potato genotypes was designed in which one of the more successful previous sucrose/glycerol protocols was modified involving a stepwise decrease in sucrose concentrations following freezing and thawing (see section 5.5).

The results of the first experiment revealed that none of the shoot buds from either of the genotypes survived the two-step freezing process, even though, these protocols involving stepwise in sucrose concentrations up to 0.7 M or 1.0 M for 8 or 10 d had given some of the best results with frozen embryogenic tissues. However, shoot buds of both genotypes survived with the dehydration alone, with the 3h period producing higher survival rates (85 – 90%).

The results of the second study showed that some of the encapsulated shoot buds survived after evaporative dehydration followed by the two-step freezing process (10 – 14% of healthy shoots). The successful protocols involved stepwise increase in the sucrose concentrations from 0.1 M (2d) to 0.15 M (3d) before a final treatment with a mixture of sucrose and glycerol (0.4 M sucrose + 2 M glycerol) (see section 5.4). In the

previous experiments, shoot buds treated with sucrose alone had not survived the two-step freezing process. Evaporative dehydration improved the survival of the frozen shoot buds, with the 5h period (19 – 27% moisture content) producing the highest survival rates. Two to three weeks after culture on MS medium supplemented with 1 μM GA3 + 0.2 μM NAA and 0.1 M sucrose, 29% of the shoot buds had shown signs of survival, with 14% producing healthy shoots, 10% forming callus on the sucrose of the shoot buds before turning brown and 5% showing abnormalities.

The results of the third experiment indicated that some shoot buds from all of the genotypes survived the two-step freezing process, and the highest survival rate of 48% was achieved with one of the four genotypes, with 33% of the surviving shoot buds producing normal shoots. Evaporative dehydration again improved the survival rates of the frozen tissue, with the best results being obtained with the 5h period (18 – 27% moisture contents). Compared with the previous experiment, the treatment on this occasion involved a more gradual decrease in the sucrose concentration following thawing but this did not improve the survival of the frozen shoot buds.

In the most successful treatment, the thawed buds were transferred directly to 0.1 M sucrose for recovery (Plate 6).

In previously published work concerning sweet potato, Towill and Jarret (1992) reported that shoot-tips from two sweet potato genotypes survived a cryopreservation sequence involving the more conventional cryoprotectants glycerol, DMSO, ethylene glycol, and which was designed to induce vitrification in the tissues. The mean survival rates of rapidly frozen shoot-tips was only 26% and moreover survival was reported to be associated with callus formation; the authors also stated that shoot-tips did not survive a

two-step freezing process. Later, deGoes (1993) also reported that shoot-tips from these sweet potato genotypes did not survive a two step-freezing process, using 10% DMSO as cryoprotectant. To induce dehydration tolerance in the encapsulation-dehydration technique as used in the present study, pre-culture alginate-coated meristems have been subjected to high sucrose concentrations (0.7 M to 1.0 M) for 16 to 24 h (Dereuddre et al, 1990; Nino and Sakai, 1992). In addition treatment with a mixture of 1.6 or 2.0 M glycerol and 0.4 M sucrose was reported to be promising in inducing dehydration prior ro freezing of navel orange meristems (Sakai et al, 1991) and it also seemed essential for producing a high survival rates, with meristems of Wasabi cooled to –196 ^{0}C (Matsumoto et al, 1995). In the present study, the approach was investigated for sweet potato shoot buds by the use of these treatments involving various concentrations of sucrose and glycerol (see section 5.5 for details), prior to evaporative dehydration, followed by the two-step freezing process. The treatment involving stepwise increase in sucrose (0.1 M to 0.15 M for 2 – 3d) and a mixture of sucrose and glycerol (0.4 M sucrose + 2 M glycerol for 18 h) seemed to be the most effective for shoot bud survival.

Following the partial success of the series of experiments involving pre-treatments with sucrose/glycerol mixtures and evaporative dehydration prior to two-step freezing, attempts were made with this basic procedure, to improve the survival rates beyond the highest value of 48% that had already been achieved. First of all, because of the influence that bulk is known to have on the freezing process, an experiment was carried out with different sizes of buds (see section 5.6). This was followed by a series of experiments in which modifications were made to the sucrose/glycerol cryopreservation mixture, involving the addition of mannitol alone (see section 5.7) or a mixture of mannitol and proline (see section 5.8).

The results of the first study indicated that with the two different sizes of explants, shoot buds from two sweet potato genotypes survived the two-step freezing process with rates in the range 5-19%. The smaller bud size (0.2-0.5mm in length) was found to be more tolerant of freezing, producing higher survival rates (14-19%) than the larger bud size (5-10% survival). Among the dehydration times, the 5h period (25-33% moisture content) was found to be the most effective for survival of frozen buds. In subsequent experiments therefore, the smaller buds were routinely employed.

The results of the second study showed that none of the shoot buds survived the two-step freezing process following treatments with stepwise increase in sucrose (0.1 to 0.15M for 2-3d) then transfer to 1M mannitol of 1M glycerol for 18h exposure, prior to dehydration and two-step freezing process.

Similarly, the results of the third study showed that none of the shoot buds survived the two-step freezing process following cryopreservation treatments involving stepwise increases in sucrose (0.1M-0.15M for 2-3d) and treatments with mixtures of sucrose, glycerol, mannitol and proline (1.0M; 0.5M; 39g/l; 5g/l respectively).

The possible cryoprotective mechanism of action of mannitol and proline are not fully understood, although mannitol can be expected to act as a non-penetrating osmotic agent (see section 1.3.1.2). As far as the sweet potato shoot meristems were concerned, however, there was no evidence of any beneficial effect resulting from the addition of either compound from the cryopreservation sequence before freezing.

No previous work concerning the effects of bud size on the cryopreservation process has been reported for sweet potato. In work with other species, such as Taro *(Colocasia esculanta, L.)*, Takagi et al (1997) evaluated three different sizes of explants and their

sensitivity to a mixture (PVS2) containing 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) DMSO prior to a rapid-freezing process. They reported that the smaller size shoot-tips (0.8mm) gave the highest survival (25.8%) after exposure to PVS2 for 20 min and prior to freezing, while the survival of the larger explants (axillary buds and 2.0mm long shoot-tip) were no higher than 5%.

Similarly, there have been no previous reports of work concerning the cryopreservation effects on sweet potato shoot buds of a combination of sucrose/ glycerol/ mannitol/ proline. However, in work with cell suspension culture of other species such as *Capsicum annuum* and *Acer pseudoplatanus* the use of mannitol was beneficial as reported by Withers and Street (1977b). Similarly, mixtures of proline and sucrose were used with some success with carnation shoot apices (Umera, 1981) as more mixtures of sucrose and glycerol with *Lilium japonicum* (Matsumoto et al, 1995) (see section 1.3.1.2 and 1.3.3 for details).

By this stage in the investigation with the shoot buds, it had not been possible to improve on the highest survival rates of 33% that had been achieved following sucose/ glycerol-dehydration cryoprotection treatment combined with the two-step freezing process. It was therefore decided, in the remaining time, to investigate an alternative approach involving cryoprotectant mixtures that have been employed by other workers to encourage vitrification when used in association with a rapid-freezing process (see chapter 1 Section 1.3.3 for further details). Those procedures are sometimes described as vitrification techniques but this is perhaps misleading since it is now known that the sucrose-dehydration techniques, as used already in the present investigations can also produce the state of vitrification (Harding et al, 1997; Englemann et al, 1992). The so called

vitrification procedures however, do at least represent a rather different approach in which high concentrations of some of the more conventional cryoprotectants such as DMSO are employed instead of high sucrose concentration. The next series of experiments (see Section 5.9-5.10), therefore, were concerned with the effects of different exposures to a cryoprotectant mixture (PVS2) containing 30% (v/v) glycerol, 15% (v/v) ethylene glycerol, 15% (v/v) DMSO on the survival of rapidly frozen encapsulated and non-encapsulated shoot buds from two sweet potato genotypes.

The results of these studies (see section 5.9 and 5.10) demonstrated that none of the encapsulated or non-encapsulated shoot buds survived the rapid-freezing process, following any of the PVS2 treatments. With the non-frozen controls, however, both the encapsulated and non-encapsulated shoot buds survived the dehydration process in combination with PVS2 treatments, indicating indirectly that the treatments in themselves were not toxic. It was not possible, therefore, that a more comprehensive investigation with this alternative approach might lead to some success.

Since it is desirable that cryopreservation protocols, if they are not to be used routinely for germplasm storage, should not be too demanding technically, a final experiment was carried out. This used more easily isolated nodal segments of sweet potato instead of shoot buds used in all of the previous experiments. The nodal segments were subjected to sucrose evaporative cryoprotective treatment in combination with rapid-freezing process, but no survival was achieved. This experience, together with the act that there have been no previous reports of the successful cryopreservation of intact nodes, would perhaps suggest that such tissues are too bulky to respond uniformly to the freezing process. If that is the case, it is quite possible that surviving tissue, even if they exist, would

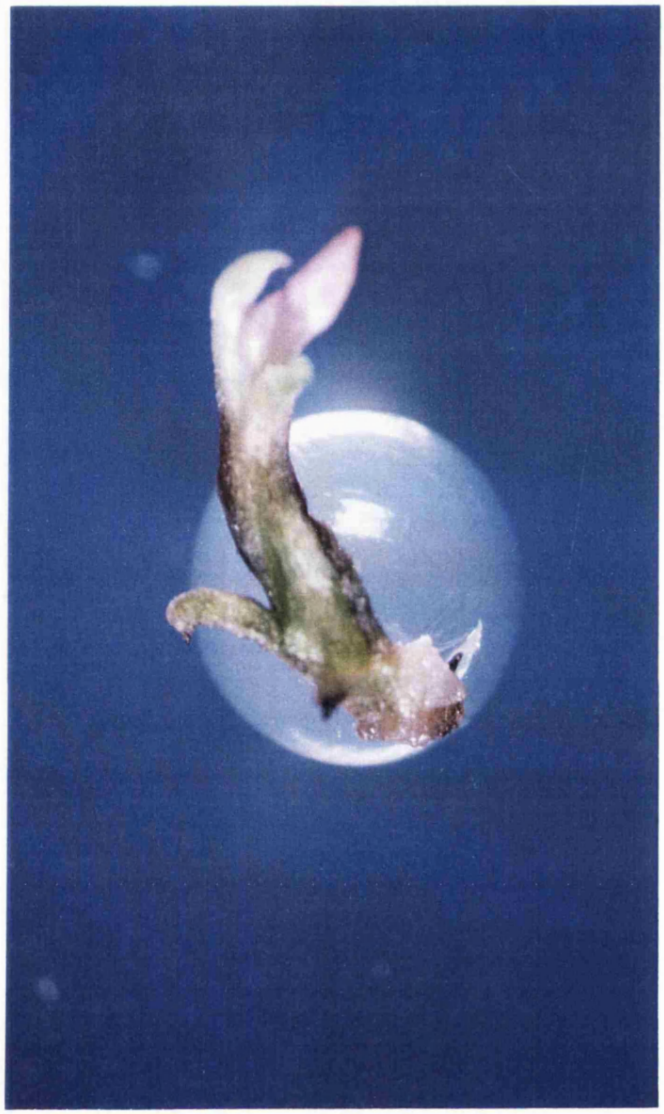
succumb to the effects of the toxic substances that are likely to be released from surrounding damaged tissues.

With regard to the use of cryopreservation techniques for the routine storage of sweet potato shoot meristems, it might be concluded that the encapsulation technique in combination with sucrose-evaporative dehydration protocols and two-step freezing have shown some promise, and that further investigations might prove their value with a wide range of genotypes. These techniques are routinely easy to handle but it would certainly be an advantage if a rapid-freezing process could be substituted for the two-step freezing sequence.

Plate 6: Survival of encapsulated shoot bud of genotype 865M after two-step freezing process

Shoot bud pre-cultured on a series of MS media supplemented with 1μM GA_3 + 0.02μM NAA and stepwise increases in sucrose concentration (up to 0.15M) for 5d; treated with a mixture of 0.4M sucrose and 2M glycerol for 18h; dehydrated for 5h and frozen by two-step cooling process; transferred to MS medium supplemented with 0.1M sucrose for 48h in dark followed by 48h in the light at 25°C. (x10)

6.



6. GENERAL CONCLUSION

6.0 GENERAL CONCLUSION

It was concluded that an efficient system for the induction and maintenance of embryogenic tissues and for subsequent plant recovery was successfully developed for all of the eight sweet potato genotypes that were tested. This system was therefore used routinely in a series of cryopreservation studies. Both the bud location and the type of auxin were found to be critical for the successful induction of embryogenic tissues. Among the three auxins tested (2,4-D; 2,4,5-T and Picloram), 2,4-D was the most effective for induction and maintenance of embryogenic cultures. Further improvements at the plant recovery stage might be achieved by the use of activated charcoal to reduce further the concentration of growth regulators in the maturation medium and also by a reduction in the strength of the basal medium during the plantlet development stages. If it is important to recover larger numbers of plantlets, competition between embryos at the maturation stage might be reduced by the further disaggregation of the embryogenic tissue in shaken liquid medium.

As far as the cryopreservation of encapsulated embryogenic tissues of sweet potato is concerned, the two-step freezing process seems to be reliable in terms of the high survival rates that were obtained with six of the eight sweet potato genotypes. With further adjustments of sucrose levels and dehydration times, it is likely that improvements could be made in the lower survival rates obtained with the remaining two genotypes. Some success was also achieved with the rapid-freezing procedure, in combination with the use of non-encapsulated embryogenic tissues. Since this technique has considerable advantages in terms of technical simplicity, it would be important to conduct further work aimed at improving its reliability with a wider range of genotypes.

Such investigation could involve changes in the sucrose pre-treatment and/ or the dehydration procedures.

These cryopreservation protocols could have an important role to play in sweet potato improvement programmes which utilize high quality embryogenic tissues for the production of transgenic plants, since they would eliminate the need for labour intensive maintenance programmes. Furthermore, the combination of somatic embryogenesis, synthetic seed technology (encapsulation) and cryopreservation is potentially a useful approach to the genetic conservation of recalcitrant species, for which storage and shoot-meristem culture techniques are not a practical proposition, especially, if it can be demonstrated that the embryogenic cultures are free from somaclonal variation.

Some success was also achieved in a group of cryopreservation experiments involving encapsulated shoot buds of four sweet potato genotypes. In this pre-treatment prior to evaporative dehydration and a two-step freezing process. An alternative approach involving cryoprotectant mixtures which had been employed with sweet potato meristems by other workers (Towill and Jarret, 1992) in association with a rapid-freezing process was not successful with either encapsulated or non-encapsulated shoot buds. In view of the importance that might be attached to the development of efficient procedures for the cryopreservation of shoot buds of sweet potato in relation to genetic conservation programmes, it can be recommended that further investigations should now be carried out to optimise the sucrose/ glycerol and encapsulation / evaporative dehydration protocols. As with the embryogenic tissues, it would be an advantage if these protocols could be developed in combination with a rapid-freezing process.

7. LITERATURE CITED

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Appendix 1

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Genstat 5  Release 3.2  (Sun/Unix)                Mon Jul 28 10:14:25 1997
Copyright 1995, Lawes Agricultural Trust (Rothamsted Experimental Station)

1  "
2     2,4,5-T
3  "
4  units [72]
5  factor geno, conc
6  open 'e1.dat';2
7  read [ch=2] geno, conc, emb, nonemb, n, perc; frep=labels, levels

Identifier   Minimum      Mean   Maximum    Values   Missing
       emb     0.000     2.306     7.000        72         0
    nonemb    0.0000    0.7500    2.0000        72         0
         n     8.000     8.000     8.000        72         0
      perc      0.00     28.82     87.50        72         0

Identifier    Values   Missing    Levels
     geno        72         0         8
     conc        72         0         3

8  model [dist=binomial] emb; nbinomial=8
9  terms conc*geno
10 fit [print=*] conc
11 add [print=*] geno
12 add [print=acc] conc.geno
```

```
..........................................................................

* Regression Analysis *****

Accumulated analysis of deviance ***

ge                                                    mean  deviance
                          d.f.     deviance     deviance     ratio
nc                           2      36.4342      18.2171     18.22  ***
o                            7      49.7621       7.1089      7.11  **
nc.geno                     14      37.2499       2.6607      2.66  **
dual                        48      19.4959       0.4062

                            71     142.9421       2.0133
SSAGE: ratios are based on dispersion parameter with value 1

  tabulate [print=means; margins=yes; class=geno, conc] emb

                Mean
     conc       1.00         5.00         10.00         Mean
     geno
   Papota      4.000        4.000        3.667        3.889
    TIB10      0.333        2.333        2.333        1.667
    1023m      2.000        4.667        3.333        3.333
     132m      0.667        0.333        2.000        1.000
     207m      0.667        3.000        3.000        2.222
     209m      1.000        6.333        2.667        3.333
     30mt      0.000        1.667        1.667        1.111
     865m      0.000        2.333        3.333        1.889

     Mean      1.083        3.083        2.750        2.306

  stop

***** End of job.  Maximum of 5853 data units used at line 14 (312904 left)
```

Appendix 2

Plant Cell Reports (1997) 16: 710–714

S. Al-Mazrooei · M. H. Bhatti · G. G. Henshaw
N. J. Taylor · D. Blakesley

Optimisation of somatic embryogenesis in fourteen cultivars of sweet potato [*Ipomoea batatas* (L.) Lam.]

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Abstract Culture procedures have been developed to facilitate the induction and maintenance of somatic embryogenic tissues in 14 out of 16 tested cultivars of sweet potato [*Ipomoea batatas* (L.) Lam]. Both the size of the axillary bud explant and the type of auxin were found to be critical for the successful induction of somatic embryogenesis. Of the five auxins screened 2,4-dichlorophenoxyacetic acid 2,4-D and 2,4,5-trichlorophenoxyacetic acid were the most effective, with use of the latter inducing the production of embryogenic tissues in 7 cultivars which responded poorly or not at all to 2,4-D. Procedures for secondary/cyclic embryogenesis, formation of mature embryos and their conversion to plants are also described.

Key words Sweet potato · Somatic embryogenesis · Auxin

Abbreviations *2,4-D* 2,4-Dichlorophenoxyacetic acid · *MS* Murashige and Skoog (1962) medium · *2,4,5-T* 2,4,5-Trichlorophenoxyacetic acid

Introduction

Somatic embryogenesis offers significant potential for the improvement of sweet potato (*Ipomoea batatas*) through the generation of tissues for use in genetic transformation programmes, such as those concerned with resistance to potyvirus complex (Newell et al. 1995; Zheng et al. 1996). It may also be important for mass clonal propagation (Bieniek et al. 1995), synthetic seed production (Chee and Cantliffe 1992) and germplasm conservation (Blakesley et al. 1995; Litz and Gray 1995).

A series of reports has been published which detail the induction of embryogenic tissues from shoot tips of the sweet potato cultivar White Star. the subsequent proliferation of these tissues in semi-solid and liquid media and the regeneration of plants (Liu and Cantliffe 1984; Chee and Cantliffe 1989; Chee et al. 1990). More recently, somatic embryos have also been produced and regenerated from petiole and leaf explants of the cultivar PI 318846-3 (Zheng et al. 1996). In all cases, however, evidence for the induction of somatic embryogenic tissues at high frequencies has been restricted to one or a few cultivars, and when attempts were made to extend this into a wide range of cultivars, the majority were found to be recalcitrant or to respond at low frequencies, generally less than 20% (Cavalcante Alves et al. 1994; Desamero et al. 1994; Zheng et al. 1996).

In order for this important tropical root crop to be improved through the application of biotechnology, it is essential that culture systems are developed which allow somatic embryogenesis to be effectively induced and maintained in as wide a range of cultivars as possible. Here we report the importance of auxin type and explant in the production and maintenance of embryogenic tissues in 14 out of 16 tested cultivars of sweet potato obtained from different geographical collections.

Communicated by M. R. Davey

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Materials and methods

Plant material

Sweet potato cultivars were obtained from a number of sources: Jewel, Sunny, Imby, Nemanete, Papota and TIS-2498 from the Centro International Potato, Peru; TIB 10 from the International Institute of Tropical Agriculture, Nigeria; Brondal from P. Kessler, South Africa; Rose Centennial and Jersey Orange from the United States De-

nent of Agriculture, Beltsville, USA, and the Chinese cultivars T, 865 M, 1023 M, 209 M, 207 M and 132 M from P. Lepoiv-embloux, Belgium.

re media and conditions

shige and Skoog (1962) (MS) basal medium was used in all ex-nents, supplemented with 0.06 or 0.09 M sucrose in addition to us types and concentrations of auxins. These were added prior usting the pH to 5.8 with 1 M NaOH, the addition of 7 g l^{-1} agar id no. 3) and autoclaving. Media were dispensed at 25 ml per Petri dish or 35 ml per 170-ml screw-cap glass jar. All cultures incubated in a growth room at 26±1°C with a 16-h photoperi-50 µmol m^{-2} s^{-1} PAR.

hoot cultures were grown in glass jars containing MS medium lemented with 0.06 M sucrose and were propagated by transfer-single node cuttings to fresh medium every 6–8 weeks. Dormant ary buds, 0.5–1.0 mm in length were used as explant material he induction of embryogenic cultures. They were excised from t cultures using a hypodermic needle and transferred to Petri es containing MS medium supplemented with 0.09 M sucrose various types and concentrations of auxin. Embryo maturation plantlet regeneration took place on MS medium containing M sucrose without growth regulators. Somatic embryo-derived lets were transferred to a 1/1 Perlite/peat mixture once they had lished a root and shoot system, and incubated in a growth cham-t 28°C for 4 weeks before being transferred to the glasshouse grown to maturity in 15-cm-diameter pots containing Levington otting compost.

he statistical significance of the differences between treatments tested using chi-square analysis.

ults and discussion

eries of experiments was undertaken to examine the ef-s of the type and concentration of auxin, and the ex-t on the production of embryogenic tissue from 16 cul-rs of sweet potato. Embryogenic tissues were identi-by the production of organised smooth and compact ctures varying from the globular to early torpedo es. Although the frequencies varied considerably be-en cultivars, embryogenesis was successfully induced 4 out of the 16 cultivars tested, and in 8 of these, more n 50% of the axillary bud explants produced embryo-ic tissue after 4 weeks culture on induction medium ble 1). This contrasts with the extensive studies carried by Cantliffe and co-workers, which were restricted to cultivar White Star.

2,4-Dichlorophenoxyacetic acid (2,4-D) and 2,4,5-tri-lorophenoxyacetic acid (2,4,5-T) were found to be hly effective for inducing embryogenesis but the opti-m type and concentration of auxin was specific to the dividual cultivars (Table 1). In 10 of the 14 responding ltivars, a significant difference ($P<0.05$) was recorded tween the effectiveness of the two types of auxin. Fur-rmore, the 4 cultivars, Jewel, Sunny, Brondal and TIS-98, which were totally recalcitrant when exposed to 4-D, could be induced to undergo embryogenesis after lture on medium containing 2,4,5-T. Concentrations of 4-D and 2,4,5-T above 10 µM (results not shown) pro-oted the formation of minimal non-embryogenic callus hich turned brown and died within 21 days. The use of

Table 1 Effect of auxin type and concentration on the induction of somatic embryogenesis from in vitro axillary buds of 16 cultivars of sweet potato. Axillary bud explants, 0.5–1 mm in length were cultured for 4 weeks on MS medium supplemented with 0.09 M sucrose and auxin. *n*=25 (*2,4-D* 2,4-dichlorophenoxyacetic acid, *2,4,5-T* 2,4,5-trichlorophenoxyacetic acid)

Cultivar	Embryogenic response (%)					
	2,4-D			2,4,5-T		
	1 µM	5 µM	10 µM	1 µM	5 µM	10 µM
30 MT	0	52	58	0	21	20
865 M	4	56	55	0	29	41
1023 M	16	56	19	25	58	24
209 M	8	8	17	12	67	35
207 M	16	25	21	8	37	37
132 M	4	0	0	8	5	24
Papota	0	46	31	50	52	45
TIB 10	0	40	64	4	28	28
Nemanete	20	48	20	60	80	52
Jewel	0	0	0	8	32	24
Imby	0	20	0	13	63	20
Sunny	0	0	0	0	13	30
Brondal	0	0	0	0	12	12
TIS-2498	0	0	0	0	0	8
Rose Centennial	0	0	0	0	0	0
Jersey Orange	0	0	0	0	0	0

Table 2 Effect of axillary bud location on the induction of somatic embryogenesis in the sweet potato cultivars TIB 10 and Nemanete after 4 weeks culture on embryogenic induction medium. Axillary buds were excised and numbered basipetally to the eighth node below the apical meristem. Buds were cultured on MS medium supplemented with 0.09 M sucrose and 5 µM 2,4-D (TIB 10) or 2,4,5-T (Nemanete). n=23–40

Nodal position	Embryogenic response (%)	
	TIB 10	Nemanete
1	50	68
2	60	65
3	53	68
4	49	68
5	68	53
6	52	52
7	48	48
8	43	43

1 µM auxin was also relatively ineffective, favouring instead the production of large amounts of non-embryogenic callus in all cultivars except Nemanete. The auxins 1-naphthaleneacetic acid and picloram (at 1–10 µM), and dicamba (at 10–200 µM) were also investigated (results not shown). Although some embryogenic response was observed, especially from medium supplemented with picloram, the frequencies were always considerably lower and the quality of tissues poorer than those produced with 2,4-D or 2,4,5-T. The use of different types of auxin has proved to be important in achieving embryogenesis in a number of species, such as dicamba for banana (Novak et al. 1989), picloram for cassava (Taylor et al. 1996) and 2,4,5-T for

chickpea (Sagare et al. 1993). In this study, employing 2,4,5-T led to the induction of embryogenic tissue in 7 cultivars which responded very poorly or not at all to 2,4-D. At this time, however, only empirical experimentation can be used to determine which of these two auxins will be superior for any given cultivar.

The size of the explant was also found to have a critical effect on somatic embryogenesis. Axillary buds were excised from the cultivar TIB 10 and separated into three size ranges: less than 0.5 mm, 0.5–1.0 mm and 1.1–2.0 mm in length, and cultured on MS medium supplemented with 5 μM 2,4-D. Only those between 0.5 and 1 mm were capable of producing embryogenic tissues at high frequencies, with the smaller and larger explants responding at less that 10%. Buds greater than 1 mm in length tended to form non-embryogenic green callus, while those under 0.5 mm failed to grow or develop in any manner. The implications of this are that as long as the correct axillary bud is used, each in vitro plantlet can yield numerous explants rather than just the one apical meristem, as used by Cantliffe and co-workers (Liu and Cantliffe 1984; Chee and Cantliffe 1989), thereby reducing the time, labour and space necessary to establish and maintain large numbers of in vitro plantlets prior to any research effort.

To determine the effect of the location of the explant on its competence to produce embryogenic tissues, axillary buds, 0.5–1.0 mm in length, were excised from in vitro shoots of the cultivars TIB 10 and Nemanete and exposed to embryogenic induction medium for 4 weeks (Table 2). Eight locations (positions 1–8) representing increasing distances from apices were investigated and it was found that in both cultivars, although the embryogenic response declined below nodal position 5, no significant differences in embryogenic potentials of explants existed between those derived from axillary buds at the different locations.

After 4 weeks culture on the induction medium, embryogenic tissues from the 14 responsive cultivars were split into pieces 2–3 mm in diameter and subcultured onto MS medium supplemented with 5 μM 2,4-D or 2,4,5-T in order to promote secondary embryogenesis and to establish continuous embryogenic cultures. After three such 4-weekly subculture cycles, the tissue was assessed for its growth and appearance. Although embryogenic tissues from all cultivars increased over the subculture period, the production of secondary embryogenic tissue varied between the cultivars (Table 3). Generally, those which responded at high frequencies to the embryogenic induction medium, for example TIB 10, Nemanete, Papota and 1023 M, tended to grow most rapidly, at least doubling over the 4-week subculture cycle. Conversely, TIS-2498 and Sunny increased by only 25% over the same period. Those cultivars producing less embryogenic tissue generally tended to form greater amounts of non-embryogenic friable callus. The type of auxin found to be most effective for promoting secondary embryogenesis varied between the cultivars and in most cases that which was most effective in the first stage also proved to be so for secondary and continuous embryogenesis. Exceptions to this were 209 M, 207 M and 132 M which, although initiated best on medium containing 2,4,5-T, grew and proliferated to a greater extent when cultured on 2,4-D.

Table 3 Production of secondary embryogenic tissues from 14 cultivars of sweet potato after routine subculture of 2- to 3-mm-sized embryogenic aggregates onto MS medium containing 5 μM auxin, every 4 weeks (+=1–2 mm, ++=2.1–4 mm, +++=4.1–6 mm)

Cultivar	Auxin	Development after 4 weeks		
		Increase in embryogenic tissue (%)	Colour of embryogenic tissue	Degree of non-embryogenic callus formation
30 MT	2,4-D	100	Yellow	+
865 M	2,4-D	150	Red/purple	+
1023 M	2,4-D	150	Red/purple	+
209 M	2,4-D	100	Yellow	+
207 M	2,4-D	100	Yellow	++
132 M	2,4-D	50	Pale yellow	+++
TIB 10	2,4-D	150	Red/cream	+
Papota M	2,4-D	75	Yellow/red	+
Nemanete	2,4,5-T	100	Yellow	+
Sunny	2,4,5-T	25	Yellow	++
Imby	2,4,5-T	75	Pale yellow	+++
Jewel	2,4,5-T	50	Pale yellow	++
Brondal	2,4,5-T	50	Yellow	+
TIS-2498	2,4,5-T	25	Yellow/red	+

In addition to the growth rate, the quality of the embryogenic tissues produced also correlated with the induction frequencies. Thus, high-quality embryogenic tissues (as assessed by the production of compact, smooth embryogenic structures with little associated formation of non-embryogenic callus) were produced in the greatest amounts by TIB 10, Nemanete 1023 M, 865 M, 209 M and 30 MT. The embryogenic tissues produced by the different cultivars varied in colour from either pale to deep yellow or were a striking deep red/purple colour (Table 3). Embryogenic callus lines in all 14 cultivars have been maintained by a repetitive 4-weekly subculture cycle for 12 months without any apparent loss of embryogenic qualities.

It is our experience that there is a correlation between the frequency of embryogenic induction, the quality and quantity of the embryogenic tissues so produced and the ability to regenerate plantlets. Achieving high induction frequencies, therefore, improves the efficiency of the whole embryogenic process, with more embryogenic tissues being generated, and plantlets recovered, in less time from less explant material. Manipulation of the culture conditions has resulted in the production of embryogenic tissues from the majority of the cultivars in this study at frequencies considerably greater than previously reported and at which they could now be employed in genetic transformation protocols (Prakash and Varadarajan 1992).

The production of mature embryos and their conversion to plantlets were investigated in the cultivars TIB 10, Nemanete and Papota by taking pieces of embryogenic tissue 2–3 mm in diameter from induction media, and subculturing onto MS medium supplemented with 0.09 M sucrose only. After 8 further weeks, these were scored for the formation of green cotyledon-stage mature embryos prior to

ble 4 Regeneration of ma- embryos and plantlets from bryogenic aggregates 3 mm in diameter) of the et potato cultivars TIB 10, ota and Nemanete, subcul- d from auxin medium onto supplemented with 0.09 μM ose. After 8 weeks, mature ryos were counted and sferred to fresh medium. r a further 8 weeks these e scored for germination plantlet formation. Results the mean ± SE

Cultivar	Time on embryogenic induction medium (weeks)	Response		
		Explants forming mature embryos (%)	Mean number of embryos per explant	Mean number of regenerated plants per explant
TIB 10	2	96	1.1 ± 0.1	0
	3	100	1.3 ± 0.1	0
	4	97	2.3 ± 0.4	0
	8	100	18.5 ± 0.9	2.8 ± 0.4
Nemanete	2	80	15.0 ± 1.1	5.6 ± 0.6
	3	72	13.0 ± 1.1	4.6 ± 0.5
	4	88	15.0 ± 0.8	4.6 ± 0.4
	8	95	17.1 ± 0.7	8.8 ± 0.8
Papota	4	100	1.7 ± 0.3	0
	8	100	9.8 ± 0.6	2.4 ± 0.3

culture onto fresh medium of the same type. Eight eks later they were assessed for conversion to plantlets ble 4). After the initial transfer to auxin-free medium, ery high percentage (90–100%) of the embryogenic tis- s in all three cultivars had formed at least one mature ryo. Nemanete produced approximately 15% cotyle- n stage embryos from each piece of embryogenic tissue ardless of the time of exposure to the induction medium. h TIB 10 and Papota, however, longer induction peri- were required for subsequent maturation; and large nbers of mature embryos could only be regenerated m tissues which had been cultured for 8 weeks on this t-stage medium. Likewise, the ability of the mature em- os to convert to plantlets (defined as the formation of ot and root tissues) was found to depend on the dura- n of exposure to the medium. This was seen most strik- ly in Papota and TIB 10, in which only embryos regen- ed from tissue cultured for 8 weeks on the first-stage dium had the ability to convert to plants, with 25 and %, respectively, of these embryos producing plantlets. ture embryos of Nemanete germinated and established ntlets at higher frequencies of 30–50%.

A higher frequency of plantlet regeneration was ieved when embryogenic tissue of Nemanete was ex- ed to increasing sucrose concentrations. Primary em- ogenic tissue after 8 weeks exposure to the induction dium was transferred to MS medium supplemented with M 2,4,5-T and 0.1 M sucrose for 2 days followed by sub- ture onto 0.4 M and 0.7 M sucrose at 2-day intervals reafter and finally onto MS medium containing 0.06 M rose for a further 10 weeks. Although this treatment did significantly increase the number of mature embryos ng produced, it enabled 88% of them to convert suc- ssfully into plantlets.

Essential for any biotechnological application is the ility to regenerate plants from the embryogenic tissues. contrast to the work of Chee et al. (1990) with the cul- ar White Star, the use of other growth regulators has not en shown to improve plant regeneration (Al-Mazrooei 96) and employing an increasing rather than a decreas- g sucrose gradient during embryo maturation was found maximise plantlet recovery. Regeneration from the em- bryogenic tissues remains inefficient, however, and further research is underway to maximise its potential for plant production.

In summary, somatic embryogenesis in 14 cultivars represents a considerable improvement on previous reports (Cavalcante Alves et al. 1994) and demonstrates that by correct manipulation of the culture parameters, high-frequency induction of embryogenesis is achievable in a significant range of sweet potato cultivars. For those cultivars which responded at frequencies below 30%, including Rose Centennial and Jersey Orange, which were completely recalcitrant in this study, further investigations are required. Preconditioning the explant material, the use of novel auxins and alternative sugar sources are being investigated to attempt to improve the embryogenic response and to increase our understanding of the underlying control of somatic embryogenesis in sweet potato.

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Appendix. 3

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Plant Cell Reports

Cryopreservation of non-encapsulated embryogenic tissue of sweet potato (*Ipomoea batatas*)

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ABSTRACT

Embryogenic tissue of the sweet potato (*Ipomoea batatas* (L) LAM) genotype TIB 10 was established from *in vitro* axillary shoot tips on Murashige and Skoog (1962) medium supplemented with 5 μM 2,4-dichlorophenoxyacetic acid. Embryogenic aggregates of fresh mass 9.0 - 12 mg were subjected to a rapid freezing protocol in liquid nitrogen following sucrose preculture and varying degrees of dehydration. Up to 50% of embryogenic explants survived rapid freezing after preculture on 0.4 or 0.7M sucrose only. Dehydration with silica gel to moisture contents in the range 18-41% improved the survival after cryopreservation of embryogenic tissue. Tissue dehydrated for intermediate periods exhibited poor survival. Following freezing, embryogenic tissue appeared to develop normally, retaining its competence to produce mature embryos and plantlets.

Abbreviations: BA, 6-benzyladenine; 2,4-D, 2,4-dichlorophenoxyacetic acid; MS, Murashige and Skoog (1962) medium

INTRODUCTION

Somatic embryogenic tissue has a key role to play in sweet potato biotechnology. In particular, it is the preferred system for genetic transformation programmes and it offers opportunities for micropropagation and the development of synthetic seed technology. Consequently, it is important to develop storage systems for the long-term maintenance of genetic stability and embryogenic competence in the cell lines.

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In an earlier report we described the development of a cryopreservation protocol for sweet potato embryogenic tissue based on the technique of alginate encapsulation and dehydration (Blakesley *et al.* 1995). It involved the initial preculture of encapsulated immature embryogenic tissue on a medium containing high sucrose, dehydration in the sterile airflow of a laminar flow hood followed by a two-step freezing protocol using a programmable freezer. In the present study we have developed an alternative procedure for the cryopreservation of embryogenic tissue based on a high sucrose preculture, with or without a subsequent dehydration step and without encapsulation.

MATERIALS AND METHODS

Tissue culture

Somatic embryogenic cultures were established from axillary shoot tips (0.5 - 1.0 mm in length) excised from actively growing shoot cultures of the sweet potato genotype TIB 10. The axillary shoot tips were placed on Murashige and Skoog (MS) (1962) medium supplemented with 0.06M sucrose and 5 μM 2,4-D and incubated for 8 weeks. Embryogenic tissue proliferated as hard, compact shiny globular structures, and it was routinely subcultured onto fresh MS medium containing the same concentration of auxin every 4-6 weeks. To induce embryo maturation and germination, clumps of embryogenic tissue were transferred to semi-solid MS medium supplemented with 0.06M sucrose, 50 nM BA, 5 nM 1-naphthaleneacetic acid and 15 μM gibberellic acid (GA_3) for 10 d prior to transfer to a hormone-free MS medium supplemented with 0.06M sucrose. All cultures were incubated at

°C with a 16h photoperiod at 50 µmol m^{-2} s^{-1} photosynthetically active radiation.

Cryopreservation

Embryogenic aggregates (9 - 12 mg fresh mass) were excised from proliferating embryogenic cultures and transferred to semi-solid MS medium supplemented with 0.1M sucrose for 3 d. They were subsequently transferred to MS medium supplemented with either 0.4M sucrose for 3 d, 0.7M for 3 d or 0.4M for 3 d followed by 0.7M for 3 d (Tables 1-3). In each experimental treatment, 3 replicate batches of 6 embryogenic aggregates were incubated on 25ml of medium contained in a 5cm Petri dish. Each replicate batch of embryogenic aggregates was then transferred to a filter paper carrier (2.0 x 0.5 cm) and dried in a 5cm Petri dish containing 2.0g of silica gel for up to 4 h. After dehydration, the carriers were transferred to 2 ml polypropylene cryovials (Camlab, UK) prior to rapid freezing. Freezing was achieved by plunging the cryovials directly into liquid nitrogen contained in a 2l Dewar flask. Moisture contents were estimated on a percentage fresh mass basis from 3 replicate batches of tissue dehydrated in an identical manner and weighed at 30 - 60 min intervals prior to final oven drying at 85°C overnight. Precise measurements however were difficult owing to the small, and variable mass and surface area of the explants.

Samples remained in liquid nitrogen for 1 h before they were thawed by placing the vials directly into a water bath at 35°C for 2 min. The carriers were then removed from the cryovials and transferred to semi-solid MS medium supplemented with 0.1M sucrose, where they rehydrated. Immediately after thawing the embryogenic tissue was incubated at 25°C in darkness for 48 h before the tissue was transferred to the light. Assessments of survival of embryogenic tissue were made regularly over subsequent weeks and the statistical significance between the total survival percentage in each treatment was tested using Chi-square analysis. The capacity of this tissue to produce mature embryos capable of germination was assessed using the regeneration media described above.

RESULTS

Incubation of embryogenic tissue on the high sucrose media resulted in a reduction in the moisture contents to 77-78% following incubation on 0.4M sucrose and 68-71% following incubation on 0.7M sucrose. Virtually all embryogenic aggregates survived a combination of high sucrose and evaporative dehydration, although the embryogenic competence was affected by the duration of dehydration (Tables 1-3) with a considerable reduction at very low moisture contents.

Table 1. Effect of dehydration only, and dehydration followed by rapid freezing on survival of embryogenic aggregates (9 - 12 mg) of TIB 10 precultured on MS medium supplemented with 0.1M sucrose for 3 d, followed by 0.4M for 3 d. Assessment made 4 weeks after treatment. n=18.

Dehydration time (h)	Moisture content (% FM)	Survival (%)			
		Dehydration only		Rapid freezing	
		Emb.	Non emb.	Emb.	Non emb.
0	77-78	100	0	50.0	50.0
1.0	66-68	100	0	11.1	55.6
2.0	46-54	100	0	22.2	33.3
3.0	18-28	66.7	33.3	33.3	38.9
4.0	10-18	16.7	83.3	0	72.2

Emb. = embryogenic tissue
Non emb. = reversion to non embryogenic tissue

Some of the embryogenic tissue was frozen immediately after the sucrose preculture without further evaporative dehydration. The survival of this tissue after rapid freezing was high, between 94.4 and 100%. Of this tissue, 44.4 - 50.0% of the explants retained their embryogenic competence, whilst the others proliferated entirely as a friable or mucilaginous callus (Tables 1-3). Explants dehydrated for 1 or 1.5 h exhibited a very low tolerance of freezing; the overall survival was considerably lower, and most of this tissue had lost its embryogenic competence (Tables 1-3).

Survival of embryogenic tissue following freezing improved with further dehydration to moisture contents in the range 18 - 41%, in explants precultured on MS medium supplemented with 0.7M sucrose (Tables 2 and 3). There was no significant difference ($p<0.01$) between the maximum survival of embryogenic tissue recorded with these treatments although the best survival, 88.9%, was obtained from embryogenic aggregates exposed to a more gradual increase in sucrose during the preculture phase (Table 3). Following cryopreservation, aggregates which retained their embryogenic competence produced new globular embryogenic structures from the

surface of the previous embryogenic tissue. The development of embryogenic structures was accompanied by the proliferation of a soft, friable mucilaginous callus and areas of red pigment were associated with the development of new embryogenic structures.

Table 2. Effect of dehydration only, and dehydration followed by rapid freezing on survival of embryogenic aggregates (9 - 12 mg) of TIB 10 precultured on MS medium supplemented with 0.1M sucrose for 3 d, followed by 0.7M for 3 d. Assessment made 4 weeks after treatment. n=18.

Dehydration time (h)	Moisture content (% FM)	Survival (%)			
		Dehydration only		Rapid freezing	
		Emb.	Non emb.	Emb.	Non emb.
0	68-70	100	0	44.4	50.0
1.0	54-59	94.4	5.6	5.6	5.6
1.5	42-52	94.4	5.6	16.7	5.6
2.0	25-41	100	0	55.6	44.4
2.5	18-31	88.9	11.1	61.1	38.9
3.0	14-21	61.1	38.9	27.8	50.0
4.0	7-16	16.7	50.0	11.1	72.2

Emb. = embryogenic tissue
Non emb. = reversion to non embryogenic tissue

Table 3. Effect of dehydration only, and dehydration followed by rapid freezing on survival of embryogenic aggregates (10 - 11 mg) of TIB 10 precultured on MS medium supplemented with 0.1M sucrose for 3 d, 0.4M sucrose for 3 d and 0.7M sucrose for 2 d. Assessment made 4 weeks after treatment. n=18.

Dehydration time (h)	Moisture content (% FM)	Survival (%)			
		Dehydration only		Rapid freezing	
		Emb.	Non emb.	Emb.	Non emb.
0	69-71	100	0	44.4	55.6
1.0	57-59	100	0	5.6	33.3
1.5	44-49	100	0	11.1	22.2
2.0	31-37	94.4	5.6	83.3	5.6
2.5	27-30	66.7	33.3	88.9	11.1
3.0	23-26	55.6	44.4	38.9	61.1
4.0	18-20	16.7	50.0	0	72.2

Emb. = embryogenic tissue
Non emb. = reversion to non embryogenic tissue

All embryogenic tissue which proliferated following cryopreservation appeared to be normal, and when transferred to the regeneration medium it had clearly retained its competence for maturation and germination, and plantlets were produced.

DISCUSSION

In the present paper we have demonstrated that it is possible to freeze small pieces of embryogenic tissue without using the technique, reported for a number of species (Dereuddre *et al* 1991; de Boucaud *et al* 1994) in which the embryogenic tissue is protected in an alginate bead. Furthermore, good survival following rapid freezing was obtained after preculture on a high sucrose medium without evaporative dehydration. Most published protocols using sucrose as the main cryoprotectant require extensive evaporative dehydration before good survival following freezing is obtained, and many authors do not report survival rates without such a treatment. Dumet *et al* (1993) however reported the cryopreservation of large heterogeneous clumps of oil palm somatic embryos (250 - 300 mg fresh mass) following sucrose preculture and evaporative dehydration. They also found that sucrose alone was sufficient to offer some protection to certain clones. In the present study, the moisture content of this non-dehydrated tissue immediately after preculture on sucrose was 68 - 78%, depending on the sucrose molarity. It is not clear how tissue with this high moisture content survived freezing, although it may be significant that the tissue was frozen rapidly. Other non-dehydrated tissues such as potato meristems have been shown to survive rapid freezing (Grout and Henshaw 1978).

In the present study, survival was severely reduced at the intermediate levels of dehydration. This may be a result of the inter- and intracellular water in the tissue being in a state of flux as the tissue quickly dried. Further dehydration to moisture contents of 18-41% however enhanced survival levels of embryogenically competent cells, and up to 88.9% survival was obtained. At these moisture contents it is likely that a glass transition, rather than ice crystallisation was obtained (Dereuddre and Kaminski 1992). This hypothesis could be verified using differential scanning calorimetry.

This technique may offer an alternative approach to that of 'encapsulation-dehydration' previously reported for the cryopreservation of small

embryogenic aggregates of sweet potato (Blakesley *et al.* 1995). It is technically simpler, as it does not necessitate the encapsulation of the tissue or the use of a sophisticated programmable freezer. However, without alginate protection, the tissue is more vulnerable, and the data shows that the period of evaporative dehydration is important. If the period of evaporative dehydration is too extensive following preculture on 0.7M sucrose, tissue can lose its embryogenic competence following freezing. This new technique will now be used to investigate the cryopreservation of a large range of sweet potato genotypes with, and without the evaporative dehydration step.

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Appendix. 4

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A SIMPLIFIED PROTOCOL FOR CRYOPRESERVATION OF EMBRYOGENIC TISSUE OF SWEET POTATO (*IPOMOEA BATATAS* (L) LAM) UTILISING SUCROSE PRECULTURE ONLY

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SUMMARY

Embryogenic tissue of six sweet potato (*Ipomoea batatas* (L) LAM) genotypes of differing geographic origin was maintained on Murashige and Skoog (1962) medium (MS) supplemented with 5 μM 2,4-dichlorophenoxyacetic acid. Embryogenic aggregates (9 - 12 mg fresh mass) were precultured on MS medium supplemented with up to 0.7M sucrose prior to rapid freezing in liquid nitrogen. Survival of embryogenic tissue of all the genotypes was obtained, with four of these ranging from 37% to 87%. Minor adjustments in the sucrose levels before and after freezing had differing effects on the survival of embryogenic tissue of different genotypes. Embryogenic tissue recovered after cryopreservation appeared to develop normally.

KEY WORDS: cryopreservation, embryogenic tissue, sucrose, sweet potato,

INTRODUCTION

Somatic embryogenic tissue offers considerable potential for sweet potato improvement as a target tissue for the development of genetic transformation protocols (9,10), for clonal propagation (1) and synthetic seed production (4). As the production and maintenance of high quality embryogenic and transgenic tissues requires considerable time and skill, it is important that storage systems are made available for such tissues, preferably at low cost.

A cryopreservation protocol for sweet potato embryogenic tissue would offer a safe, long term storage option, but, to be of significant use it should be simple, and applicable to a wide range of genotypes. In an earlier report, we described a 'sucrose dehydration' protocol for embryogenic tissue which was successful with the sweet potato genotype TIB 10 (3). Although the best results were obtained after evaporative dehydration to low moisture contents, some survival of embryogenic tissue was obtained without the cryoprotective dehydration step. A simplified protocol based on rapid freezing, and which does not require cryoprotective dehydration could be particularly useful. The present paper reports a wider investigation into the cryopreservation of six sweet potato genotypes of varying geographic origin using a protocol based simply on sucrose treatment and rapid freezing.

MATERIALS AND METHODS

Plant material: Shoot cultures of six sweet potato genotypes: TIB 10 (from the International Institute of Tropical Agriculture, Nigeria) Papota, (from Centro International Potato, Peru), 30MT, 865M, 1023M and 209M (Chinese genotypes from P Lepoivre, Gembloux, Belgium) were established from axillary buds, and routinely subcultured on a semi-solid MS (Murashige and Skoog) (8) medium supplemented with 0.06M sucrose. Embryogenic tissue was initiated and maintained on semi-solid MS medium supplemented with 0.06M sucrose and 5 μM 2,4-D. In the preparation of MS medium, sucrose and 2,4-D were added prior to adjusting the pH to 5.8, the addition of $7gl^{-1}$ agar (Oxoid No. 3) and autoclaving. Media were dispensed at 25ml per 9cm Petri dish or 35ml per 170ml screw-cap glass jar. All cultures were incubated in a growth room at 25±1°C with a 16h photoperiod at 50μmol $m^{-2}s^{-1}$ photosynthetically active radiation. Shoot cultures were grown in glass jars containing MS medium and propagated by transferring single node cuttings to fresh medium every 6-8 weeks. Embryogenic cultures were established from axillary buds (0.5 - 1.0 mm in length) excised from actively growing shoot cultures. Buds were placed on MS medium containing 5 μM 2,4-D and incubated for 8 weeks and embryogenic tissue was then routinely subcultured every 4-6 weeks.

Cryopreservation: In the following protocols all MS media contained 5 μM 2,4-D. Embryogenic aggregates (9 - 12 mg fresh mass) were excised from proliferating embryogenic cultures and transferred to semi-solid MS medium supplemented with 0.1M sucrose for 3 d. They were subsequently transferred to MS medium supplemented with 0.4M for either 6d or 9d, and in certain treatments, to medium supplemented with 0.7M sucrose for 3d or 6d. In each sucrose x genotype treatment, 30 embryogenic aggregates (3 batches of 10) were incubated on 25ml of medium contained in a 9cm Petri dish. Each batch of 10 embryogenic aggregates was then transferred to a filter paper carrier (2.0 x 0.5 cm) which was placed inside a 2 ml polypropylene cryovial (Camlab, UK) prior to rapid freezing. Freezing was achieved by plunging the cryovials directly into liquid nitrogen contained in a 2l Dewar flask. Moisture contents were estimated on a percentage fresh mass basis from 3 replicate batches of tissue whose dry mass was determined following oven drying overnight at 85°C. Samples remained in liquid nitrogen for 1 h before they were thawed by placing the vials directly into a water bath at 35°C for 2 min. The carriers were then removed from the cryovials and transferred to semi-solid MS medium supplemented with 0.1M sucrose, or subjected to a sequence involving incubation on MS medium supplemented with decreasing concentrations of sucrose prior to a final transfer to 0.1M sucrose. Immediately after thawing the embryogenic tissue was incubated at 25°C in darkness for 48 h before the tissue was transferred to the light. Assessments of the total survival percentage of the embryogenic tissue (30 replicates per treatment) were made regularly, and the statistical significance of the survival percentages after 6 weeks was tested using the Kruskal-Wallis test.

RESULTS

Embryogenic tissue of each of the six genotypes was induced on axillary bud explants at frequencies in excess of 50%, and was characterised by organised smooth, compact globular structures varying in colour from yellow (30MT) to red/purple (1023M). Incubation of this tissue on high sucrose media resulted in a decline in the moisture content to 77-79% on 0.4M sucrose and 70-73% on 0.7M sucrose. Incubation on high sucrose media alone did not affect the viability of the embryogenic aggregates of any of the genotypes used, and all embryogenic tissue growth appeared normal when transferred to embryogenic maintenance medium, although the actual growth rate was not determined.

Following cryopreservation, overall 'tissue' survival was high, with embryogenic tissue or non-embryogenic callus present on virtually all explants (Table 1). Aggregates of all genotypes which retained their embryogenic competence started to produce new, organised globular

Table 1. Effect of rapid freezing in liquid nitrogen on the survival of naked embryogenic aggregates of 6 sweet potato genotypes subjected to various sucrose treatments.

Genotype	Sucrose treatment	Moisture content (% fresh mass)	Survival after rapid freezing (%)	
			Embryo-genic	Non embryogenic
865M	A	78	50	47
	B	78	60	40
	C	70	83	17
	D	70	43	40
	E	71	27	63
TIB 10	A	77	43	57
	B	77	53	47
	C	70	57	43
	D	70	40	60
	E	70	57	43
1023M	A	79	13	87
	B	79	0	47
	C	71	7	93
	D	71	37	60
	E	71	0	100
30 MT	A	78	0	100
	B	78	3	97
	C	72	10	90
	D	72	0	100
	E	72	37	63
Papota	A	78	3	97
	B	78	10	90
	C	72	23	77
	D	72	7	93
	E	73	7	93
209M	A	79	3	97
	B	79	3	95
	C	72	0	100
	D	72	7	93
	E	72	10	90

30 embryogenic aggregates were used per 'genotype x sucrose' treatment

Sucrose treatment prior to freezing:	Sucrose treatment after freezing
A: 0.1M 3d, 0.4M 9d	0.4M 3d, 0.1M
B: 0.1M 3d, 0.4M 9d	0.4M 6d, 0.1M
C: 0.1M 3d, 0.4M 6d, 0.7M 3d	0.4M 3d, 0.1M
D: 0.1M 3d, 0.4M 6d, 0.7M 3d	transfer directly to 0.1M
E: 0.1M 3d, 0.4M 6d, 0.7M 6d	transfer directly to 0.1M

embryogenic structures from the surface of the original explant after a lag period of 5-10 days. This was accompanied by the proliferation of a friable or mucilaginous non-embryogenic callus. In many cases however, although aggregates survived, they had clearly lost their embryogenic competence, as only friable or mucilaginous callus was produced. The final survival percentage of embryogenic tissue, assessed 6 weeks after freezing varied with genotype, and with the sucrose treatment used; survival of TIB 10 and 865M was very high, up to 57% and 87% respectively. Thirty seven percent survival was obtained with 1023M and 30MT, but survival of the other two genotypes was only 10 - 23% (Table 1). Sucrose treatment effects were only found to be significant with genotypes 865 and 1023M (Table 1). In these cases, treatments 1-3 gave the highest survival ($P<0.05$) of embryogenic tissue of 865 whereas for 1023M, treatment 4 was the most successful ($P<0.05$). In contrast, modification

of the sucrose pretreatment did not significantly affect the survival of embryogenic tissue of the other genotypes. Embryogenic tissue appeared to develop normally following cryopreservation, and retained its ability to produce mature embryos and plants when transferred to a non-hormone medium. A time course of the actual recovery of embryogenic tissue following cryopreservation was not carried out.

DISCUSSION

It was noticed by Dumet et al (6) with oil palm embryogenic tissue, and by Blakesley et al (3) with embryogenic tissue of a single genotype of sweet potato, that survival prior to evaporative dehydration could be obtained in the 'sucrose-dehydration' protocols, where tissue was not encapsulated. Dumet et al (6) obtained relatively low survival rates ranging between 10 and 23% in four of nine clones tested. In this report we have shown that it is also possible to freeze embryogenic tissue of sweet potato with the use of sucrose alone, without any other cryoprotectant treatment. This contrasts with protocols which utilise an encapsulation and/or an evaporative dehydration step. Most cryopreservation protocols involve extensive cellular dehydration prior to freezing to the temperature of liquid nitrogen; these include freeze-induced cell dehydration (12), the 'encapsulation-dehydration' technique (7) and the 'vitrification' technique (11). Each of these protocols avoid the problem of ice-crystal formation by inducing the process of vitrification, in which water undergoes a transition to a less damaging non-crystalline 'glassy' state (5). The technique of rapid freezing following sucrose treatment described in the present paper is unlikely to involve vitrification, as the moisture content of the tissue is too high. It is likely in the present system that during freezing, homogeneous ice crystal formation takes place. Survival of embryogenic tissue of TIB 10, 865M, 1023M and 30MT was high, between 37% and 87%. However, in the case of 209M and Papota, high survival was accompanied by the loss of embryogenic competence. This could have been due to ice crystal damage caused during freezing, or through recrystalisation during thawing. Consequently further progress may be made with this technique by careful attention to cooling and warming rates. For truly recalcitrant genotypes it may be necessary to consider encapsulation and two-step freezing protocols involving evaporative dehydration (2).

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Cryopreservation of embryogenic tissue of a range of genotypes of sweet potato (*Ipomoea batatas* [L] Lam.) using an encapsulation protocol

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Abstract Embryogenic tissue of nine sweet potato [*Ipomoea batatas* (L.) Lam] genotypes from Asia, Africa and the America, was established from in vitro axillary buds on Murashige and Skoog medium supplemented with 2,4-dichlorophenoxyacetic acid or 2,4,5-trichlorophenoxyacetic acid. Embryogenic aggregates, 1.0–2.0 mm in diameter, were encapsulated in alginate gel, precultured on medium containing elevated levels of sucrose and dehydrated prior to rapid freezing in liquid nitrogen. The maximum survival of embryogenic tissue ranged from 4% to 38%, depending on the genotype. With the incorporation of a slow-cooling step, survival was generally much higher than that obtained after rapid freezing alone. Five of eight genotypes tested with this protocol gave survival percentages in excess of 55%, and a further two in excess of 33%, all after evaporative dehydration. The most effective sucrose treatment(s), however, varied with the genotype.

Key words Sweet potato · Cryopreservation · Somatic embryogenesis · Encapsulation

Abbreviations *2,4-D* 2,4-Dichlorophenoxyacetic acid · *MS* Murashige and Skoog medium · *2,4,5-T* 2,4,5-Trichlorophenoxyacetic acid

Introduction

In sweet potato, as in numerous other species, somatic embryogenic tissue offers considerable potential for crop improvement, as a target tissue for the development of genetic transformation protocols with *Agrobacterium* (Newall et al. 1995) or direct gene transfer technologies (Prakash and Varadarajan 1992). It may also be suitable for clonal propagation (Bieniek et al. 1995), and synthetic seed production (Chee and Cantliffe 1992). There are, however, a number of difficulties associated with the development of suitable embryogenic cultures, and the production of high-quality embryogenic tissue from a wide range of genotypes is a skilled task. Considerable time can be required to produce high-quality embryogenic tissues, and once produced, these elite cell lines must be maintained by frequent subculture, with the consequent risk of losses and genetic variation. It is therefore important, that storage systems are made available for such tissues which, at low cost, minimise the risk to genetic stability. Further, as genetic transformation technologies are developed and applied, there is an increasing demand for technology for the preservation and storage of transgenic cell lines.

A cryopreservation protocol for sweet potato embryogenic tissue is, therefore, important because it directly addresses these issues by offering a safe, long-term storage option. The first reported cryopreservation protocol for sweet potato embryogenic tissue (Blakesley et al. 1995) was based on encapsulation, sucrose pretreatment and evaporative dehydration prior to freezing. Good survival of embryogenic tissue was obtained with one of the two genotypes tested, and only if a two-step freezing strategy was employed (Blakesley et al. 1995). Before such a protocol can be recommended for practical use, it must be shown to be applicable to a wide range of genotypes. In the present paper we report the cryopreservation nine sweet potato genotypes from different geographical localities, and consider the importance of freezing rate, and the interaction of the genotype with differing sucrose pretreatments and dehydration times.

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Materials and methods

Plant material

Shoot cultures of nine sweet potato genotypes – TIB10 (from the International Institute of Tropical Agriculture, Nigeria) Nemanete,

pota, (from Centro International Potato, Peru), 30MT, 1023M, 65M, 132M, 207M and 209M (Chinese genotypes from P. Lebivre, Gembloux, Belgium) – were established from axillary buds d routinely subcultured on a semi-solid Murashige and Skoog 962) (MS) medium supplemented with 0.06 M sucrose. Embryonic cultures were established from axillary meristems (0.5–1.0 mm length) excised from actively growing shoot cultures. Meristems ere placed on MS medium containing 5 μM 2,4-dichlorophenoxyatic acid (2,4-D) (5 μM 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) r 209M and Nemanete), and incubated for 8 weeks. Embryogenic ssue was then routinely subcultured every 4–6 weeks onto fresh MS edium containing 5 μM 2,4-D (5 μM 2,4,5-T for Nemanete). All ltures were incubated at 25°C with a 16-h photoperiod at 50 μmol $^{-2}$ s^{-1} PAR.

ryopreservation

n the following protocol, all MS media contained either 5 μM 2,4,5- for Nemanete or 5 μM 2,4-D for all other genotypes. Embryogenc aggregates, 1.0–2.0 mm in diameter were excised from proliferatng secondary embryogenic cultures and suspended in liquid MS medium supplemented with 3% (wt/vol) sodium alginate (Sigma, UK) and 0.1 M sucrose. This mixture was dispensed with a sterile Pasteur pipette into liquid MS medium supplemented with 100 mM calcium chloride and 0.1 M sucrose. The resulting beads (4.5–5.5 mm in diameter) were removed from the liquid medium after 15 min and transferred to semi-solid MS medium supplemented with 0.1 M sucrose for 3 days, and subsequently to MS medium supplemented with higher levels of sucrose as indicated in Table 1.

In each experimental treatment, three replicate batches of ten beads were incubated on 25 ml of medium contained in a 9-cm Petri dish. Beads were then dehydrated by transferring them to an empty open Petri dish and exposing them to the sterile airflow in a laminar flow hood for up to 6 h. After dehydration, beads were transferred to a 2-ml polypropylene cryovial (Camlab, UK) prior to freezing. Rapid freezing was achieved by plunging the cryovials directly into liquid nitrogen contained in a 2-l Dewar flask. Two-step freezing was carried out as follows. The first stage involved slow cooling from ambient to 0°C at 10°C min^{-1}, and then to –40°C at 0.5°C min^{-1} in a programmable freezer (Planer, UK) followed by immediately plunging the cryovials into liquid nitrogen in a Dewar flask. Samples remained in liquid nitrogen for 1 h before they were thawed by placing the vials directly into a water bath at 38°C for 2 min. The beads were removed from the cryovials and transferred directly to semi-solid MS medium supplemented with 0.1 M sucrose, or they were subjected to decreasing concentrations of sucrose (Table 1), prior to a final transfer to 0.1 M sucrose. Irrespective of the medium, immediately after thawing the beads were incubated at 25°C in darkness for 48 h before the embryogenic aggregates were removed from the beads and incubated at 25°C in the light. The survival of embryogenic tissue was monitored over subsequent weeks, and data are presented for the final assessment made 4 weeks after the various cryopreservation treatments. Survival was determined as the proliferation of either embryogenic tissue or non-embryogenic callus. The statistical significance between freezing protocols was tested using chi-square analysis. A two-way analysis of variance of arcsine-transformed data was employed to identify significant interactions between genotype, sucrose treatment and dehydration time.

Results and discussion

Somatic embryogenesis in sweet potato

The production of embryogenic tissue on axillary bud explants of TIB10 and Nemanete has been previously described (Blakesley et al. 1995, 1996), but essentially, the embryogenic tissue of all the genotypes proliferated as hard, compact, shiny globular structures, with colour varying from deep red (1023M) to yellow (Nemanete).

Rapid freezing

An encapsulation-dehydration protocol was initially developed for embryogenic tissue of sweet potato with the genotype TIB10 (Blakesley et al. 1995). This was based on a two-step freezing protocol, as the survival following rapid freezing was poor. In the present study, the rapid-freezing protocol was investigated with nine genotypes to see whether the poor survival of TIB10 was a genotype effect. For each genotype, the same sucrose treatment (1) was employed: 0.1 M sucrose for 3 days, 0.4 M for 3 days and 0.7 M for 2 days prior to freezing, and direct transfer to 0.1 M sucrose after freezing. Survival with and without cryopreservation was monitored every hour during the 5-h dehydration period. In each genotype, survival of embryogenic tissue after 'dehydration only' declined with time, although the tolerance of dehydration stress varied to some extent with the genotype (Table 2). Maximum survival following cryopreservation ranged from just 4% of Nemanete embryogenic aggregates to 38% of 209M (Table 2). Papota did not survive freezing. Two of the genotypes, TIB10 and 865M, gave their highest survival of embryogenic tissue after preculture on the high-sucrose media without further dehydration in the laminar flow cabinet. The other genotypes required dehydration to moisture contents below 30%, but the optimum dehydration time was not consistent, varying from 3 h with 209M to 6 h with 1023M. In contrast, most published encapsulation protocols employ rapid freezing, but this did not result in acceptable survival levels of sweet potato embryogenic tissue.

Table 1 Cryopreservation treatments (*d* days)

Treatment	Sucrose treatment	
	Prior to dehydration/freezing	After dehydration/freezing
1	0.1 M 3 d, 0.4 M 3 d, 0.7 M 2 d	Transfer directly to 0.1 M
2	0.1 M 3 d, 0.4 M 3 d, 0.7 M 2 d, 1.0 M 2 d	Transfer directly to 0.1 M
3	0.1 M 3 d, 0.4 M 3 d, 0.7 M 2 d	0.7 M 2 d, 0.4 M 2 d, 0.1 M
4	0.1 M 3 d, 0.4 M 3 d, 0.7 M 2 d	0.4 M 3 d, 0.1 M
5	0.1 M 3 d, 0.4 M 3 d, 0.7 M 3 d	Transfer directly to 0.1 M

Table 2 Summary of the effect of dehydration and rapid freezing in liquid nitrogen on the survival of encapsulated embryogenic aggregates of eight sweet potato genotypes (n=21–30). The sucrose treatment prior to dehydration was 0.1 M for 3 days, 0.4 M for 3 days and 0.7 M for 2 days. After dehydration the aggregates were transferred directly to 0.1 M sucrose. *Moisture content* is for the treatment giving maximum survival of embryogenic tissue after freezing

Genotype	Moisture content (% fresh mass)	Survival (%)			
		Dehydration only		Rapid freezing	
		Embryogenic	Non embryogenic	Embryogenic	Non embryogenic
30MT	15	57	0	19	0
209M	26	57	34	38	0
1023M	12	62	5	28	20
865M	71	90	0	29	0
132M	22	38	5	5	0
207M	29	43	29	14	0
TIB10	71	100	0	29	64
Papota	42	86	14	0	0
Nemanete	19	43	58	4	53

Two-step freezing

In contrast to rapid freezing, the inclusion of a slow-freezing step prior to plunging into liquid nitrogen improved the maximum survival of TIB10 embryogenic tissue from 28% to 74% (Blakesley et al. 1995). In the present study, in five of the eight genotypes (865M, 1023M, 132M, TIB10 and Papota) tested with both freezing protocols, a significantly higher percentage survival (P<0.05) of embryogenic tissue was noted after two-step freezing in comparison to rapid freezing (Table 2, Fig. 1). For each of the eight genotypes, five different sucrose treatments were subsequently compared, and for each of these, the survival following evaporative dehydration, with and without cryopreservation, was monitored every hour during a 6-h dehydration period. For each genotype and sucrose treatment, data are presented for survival of freezing after dehydration times of 3, 4 and 5 h, after which virtually no increase in survival was obtained. The moisture contents after 3 h ranged from 19–34%, after 4 h from 14–31% and after 5 h from 11–27%.

Embryogenic tissue of all genotypes survived freezing, generally at higher levels than after rapid freezing. Overall, there was a significant genotype effect (P<0.001) on survival of two-step freezing, and also a significant interaction (P<0.001) between the genotype and the sucrose treatments. Five of the eight genotypes gave survival percentages in excess of 55%, and a further two in excess of 33%, all after 3–5 h evaporative dehydration (Fig. 1). The most effective sucrose treatment(s) varied with the genotype, and no individual sucrose treatment was significantly superior to any other. However, with the exception of 865M and 132M, sucrose treatment 3 gave good survival of evaporative dehydration and cryopreservation (Fig. 1). This treatment involved the transfer of embryogenic tissue to a high-sucrose medium immediately after freezing. It is not possible to identify an optimum length of evaporative dehydration which would give maximum survival for any genotype, as there was no significant interaction between genotype and dehydration time. With the exception of 865M, no survival of freezing was recorded without evaporative dehydration in the laminar flow cabinet (data not shown); here, 9% survival of embryogenic tissue of 865M was noted with sucrose treatment 3.

Plessis et al. (1993) also found that the best survival of grapevine shoot tips was obtained following two-step freezing. The reason for higher survival of dehydrated encapsulated tissue with the inclusion of a slow-freezing step is not understood. The two-step freezing protocol gave good survival with seven of the eight genotypes tested. However, it is not possible to identify one single sucrose treatment which could be recommended for all genotypes. Consequently, for any new genotype, a number of different sucrose treatments should be investigated, although transfer of embryogenic tissue to a high-sucrose medium immediately after freezing gave the most promising results. Sucrose has been widely reported to be necessary to induce tolerance of dehydration and subsequent freezing of encapsulated tissue (Dereuddre et al. 1990; Fabre and Dereuddre 1990), but the mechanism of sucrose action in this respect is not fully understood. Preculture on high-sucrose media results in the accumulation of sugar in the alginate bead and in the tissue (Gonzalez-Arnao et al. 1996) and this sucrose contributes to the maintenance of tissue viability during the dehydration treatment (Dumet et al. 1993). It has been suggested (Crowe et al. 1984, 1990) that the accumulation of sugar in the tissue maintains membrane stability in the dehydrated state by replacing water molecules at the charged exterior surface of membranes. In many cases, the encapsulation-dehydration protocol simply requires a short preculture period on one elevated level of sucrose; for example, Gonzalez-Arnao et al. (1996) precultured sugar cane apices on 0.75 M sucrose for just 24 h prior to dehydration and freezing, with longer preculture periods inhibiting survival after freezing. In contrast, it was found that a stepwise increase in sucrose was necessary for optimum protection of encapsulated embryogenic tissue of the sweet potato genotype TIB10 (Blakesley et al. 1995). It would also be necessary for any new genotype to freeze after different dehydration periods. It may be possible to overcome this problem by more controlled evaporative dehydration, which is currently under investigation in our laboratories.

The cryopreservation protocol described here could have an important role to play in sweet potato improvement programmes which utilise high-quality embryogenic tissue. Furthermore, the combination of somatic embryogenesis, synthetic seed technology (encapsulation) and cryopreservation is a potentially useful new approach to the genetic conservation of a recalcitrant species in its own right, particularly if it can be demonstrated that embryogenic cell cultures are free from somaclonal variation.

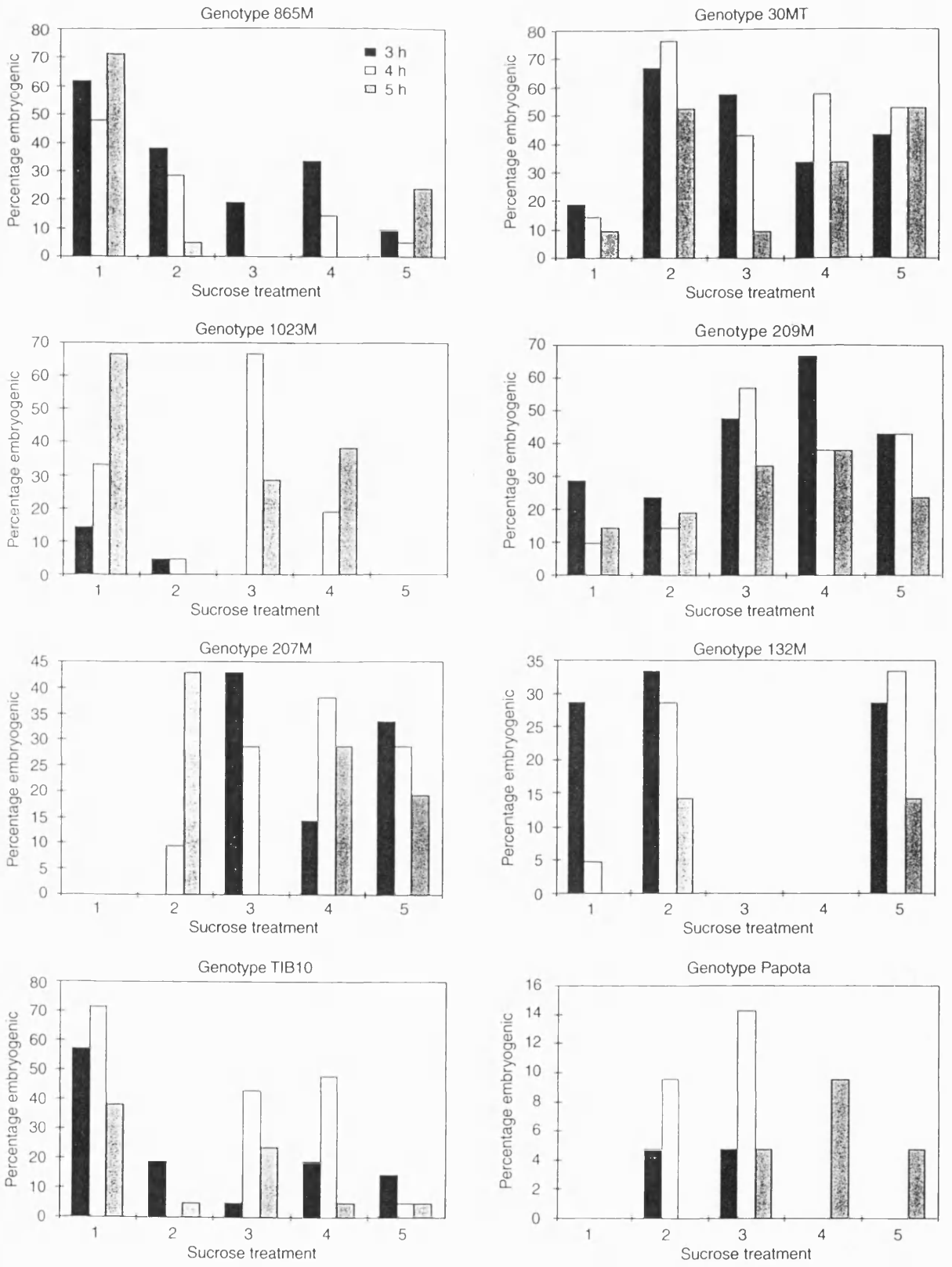

Fig. 1 Survival of encapsulated embryogenic aggregates of eight sweet potato genotypes following different sucrose preculture treatments, evaporative dehydration and two-step freezing. For each genotype×sucrose treatment (see Materials and methods), the data are presented for tissue dehydrated for 3–5 h (n=21)

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